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The Behaviour of Basophil Cytoplasmic Substances during Neural Induction in the Chick

by J. O. LAVARACK[1]

From the Department of Anatomy and Histology, University of Melbourne

WITH TWO PLATES

INTRODUCTION

ON the possible role of nucleic acids in organizer activity Brachet's (1940) histochemical researches show that a basophil cytoplasmic material, which he identifies with ribose nucleic acid, is present generally in regions of active differentiation and that it accumulates at interfaces between inductors and the cells responsive to them. These conclusions are supported by Gallera & Oprecht (1948). Further, it is known that much of the ribose nucleic acid of cytoplasm is bound to the submicroscopic particulates and certain observations suggest that neural induction (Holtfreter, 1933; Brachet, 1950) and induction of the lens vesicle (McKeehan, 1951) depend at some stage on contact or the transfer of relatively large particles from one cell group to another.

It may well be that any chemical mechanisms concerned are complex and include chains of reactions involving a number of substances. Among possible participants nucleic acids are of particular interest because of their metabolic relationships with proteins. The present investigation attempts to extend the analysis of the role of cytoplasmic basophil substances by following in some detail their behaviour in the differentiation of the cells concerned throughout the critical periods of an induction.

METHODS

Hen eggs were incubated at between 36° and 39° in an atmosphere kept moist by evaporation of water. After incubation for the desired period each egg was opened and the embryo fixed immediately by buffered 10 per cent. neutral formalin, either on the yolk or after excision with the surrounding area opaca. The embryos were passed through alcohols and xylene, infiltrated with paraffin at 56° for 2 or 3 hours and then blocked and kept at 4° until cut into sections.

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The observations to be reported are based principally on the examination of serial sections of five embryos at stages ranging from 3 to 8 (Hamburger & Hamilton, 1931).

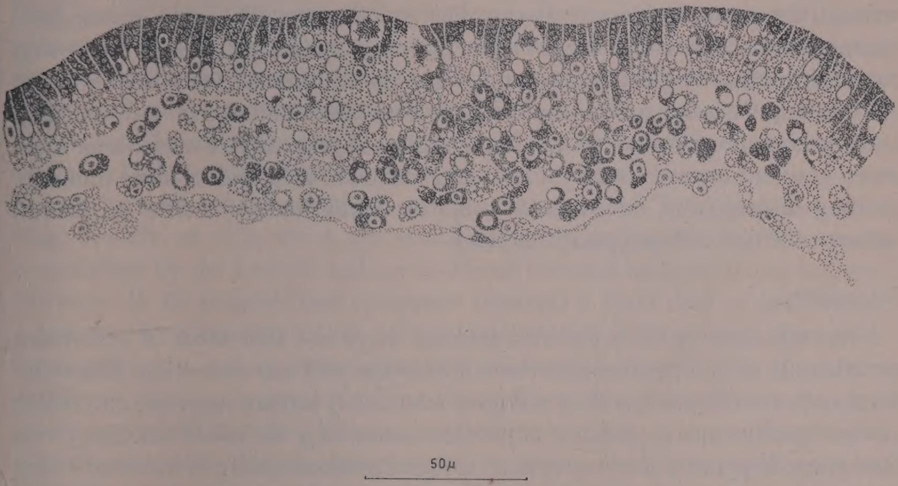
The paraffin sections were mounted by floating on water at 40–45° and dried at 40° using an albumen-glycerine adhesive. Some were stained by the methyl green-pyronin technique described by Brachet (1953), the dyes used being methyl green 'Anachemia' and pyronin 'Geigy'. Any pyronin reactivity described refers to the behaviour of material during methyl green-pyronin staining. The action of ribonuclease on cell components was tested on paraffin sections which were dewaxed in xylene and passed through alcohols to distilled water. The test sections were then incubated in a 1 in 5,000 solution of ribonuclease (salt free, crystalline, Worthington Biochemical Laboratory, Freehold, N.J., U.S.A.) at 50–55° for 60–75 minutes, according to the technique for Brachet's test described by Lison (1953), then rinsed and stained with methyl green-pyronin as before. Control sections (1) incubated with distilled water concurrently with the test section, and (2) not incubated, were stained with methyl green-pyronin in the same batch as each test section. All controls showed normal intensity of staining.

OBSERVATIONS

Pyronin-reactive cytoplasmic substance in epiblast of early primitive-streak stages

An embryo in its intermediate-streak stage (Hamburger & Hamilton, stage 3+) stained with methyl green-pyronin gave a strong basophil reaction to pyronin (Plate 1, fig. A). The intensity of this reaction may equal that of the most strongly reacting adult tissues; intensity is highest in the cells of the epiblast and primitive streak (Plate 1, fig. A). A series of tests on sections from the same embryo showed that the pyronin-reactive material is removed by preliminary treatment with ribonuclease and it is therefore provisionally identified as ribose nucleic acid. These results agree with those of Brachet (1940) and Gallera & Oprecht (1948).

In embryos at about the intermediate- or long-streak stages the epiblast is a single layer of cells which are extremely flat in the plane of the surface in the extra-embryonic zone and gradually increase in height toward the embryonic axis. At the periphery of the area pellucida the cells are of low columnar form and in the region of the primitive streak and immediately in front of it tall columnar (Text-fig. 1). The nuclear substance, excepting the nucleolus, in these and other cells examined, appears blue after methyl green-pyronin staining, apparently containing materials reactive to both dyes. After preliminary treatment with ribonuclease the nuclear substance was stained only by the methyl green component (Brachet, 1944). The nucleoli are relatively large and stained deeply by pyronin, the substance responsible being also ribonuclease soluble. In the columnar epiblast cells of the embryonic area the cytoplasm superficial to a centrally located nucleus contains more pyronin after staining than the rest of the cell. The pyronin-staining material in this zone is not homogeneous but appears to contain numbers of closely packed bodies which either stain or are enveloped in stainable substance. The cytoplasm basal to the nucleus contains large non-staining bodies of unequal sizes and this is interpreted as a zone of high yolk content; the pyronin-staining material here is distributed mainly in a light framework of substance between the non-staining bodies.

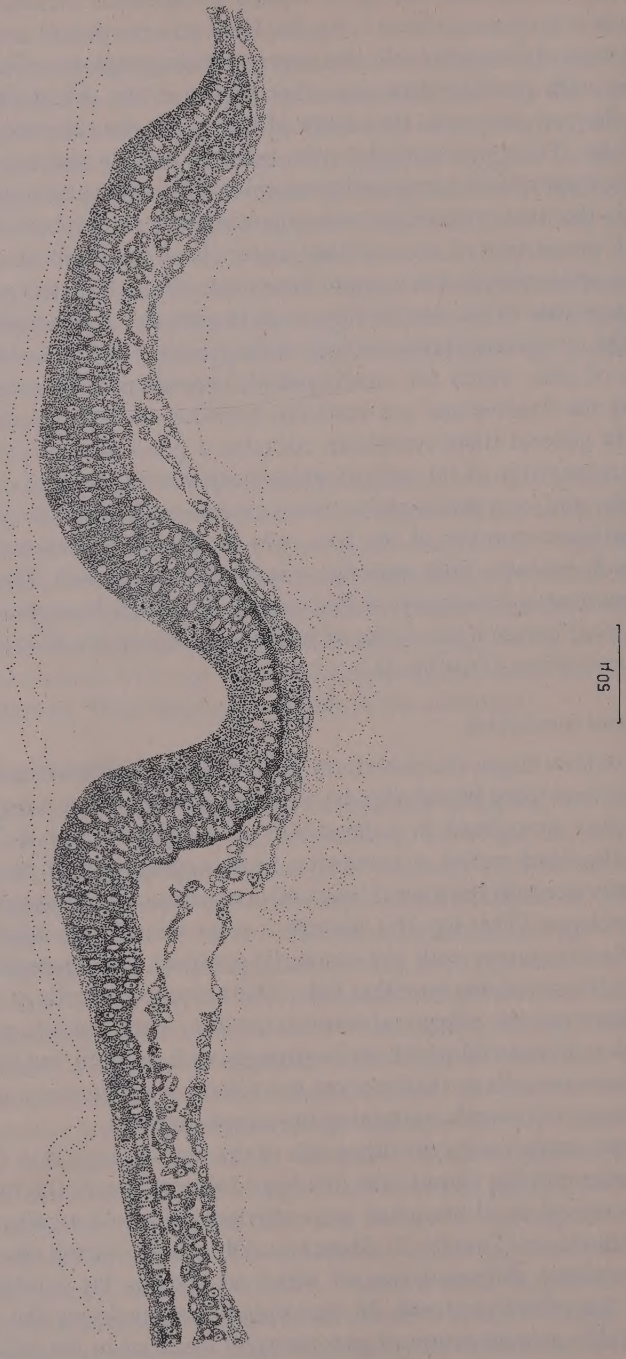


TEXT-FIG. 1. Cross-section through the anterior end of the primitive streak in the intermediate-streak stage (3+). The streak occupies a little less than the central half of the field from side to side and mesoblast is distinguishable at its inferior and lateral margins. The distribution of cytoplasmic basophil material is illustrated. Contrasts between intracellular zones have been emphasized in drawing.

In the flat epiblast cells of the extra-embryonic area no such differences between superficial and deep cytoplasm are appreciable. The cytoplasm here contains much pyronin-staining material and also numbers of non-staining bodies of a wide range of size.

Presumptive neural ectoderm and mesoderm

Maps are now available of the fate of cells in various regions of the chick embryo from primitive-streak stages onward (Wetzel, 1929; Graper, 1929; Waddington, 1932; Pasteels, 1937; and Spratt, 1946, 1947). These are in sufficient agreement to allow one to select certain cells of the epiblast as representative of presumptive mesoderm and presumptive neural tube so that, although there is still doubt as to the stage of development during which one exerts its inductive effect upon the other (and it may be before, during, or after gastrulation), study of primitive streak stages can provide information about early chemical properties of cells involved in neural induction.



TEXT-FIG. 2. Cross-section through the neural groove in the head-fold stage (6) to illustrate the distribution of cytoplasmic basophil material. There is a strong staining in the deep zone of the neural epithelium.

of cytoplasm surrounding their nuclei. Their staining with pyronin is of about the same order of intensity as that of the notochord. In the somite mesoderm, as in the notochord of this stage, the cells adhere together closely and are polyhedral after fixation. Their cytoplasm is basophil and about as intensely stained as that of the notochordal cells. It contains a high proportion of pyroninophil material and a small but varying amount of unstained substance. In this head-fold stage the somite mesoderm is still not segmented but, at the level of the future first somite, the cells are beginning to elongate and become so arranged that their inner zones converge together toward the centre of the mass, and this is accompanied by a visible change in the distribution of pyronin-staining material. A particularly high concentration of this material has now accumulated in the cytoplasm toward the dorsal surface of the developing somite. Here it forms a most intensely and homogeneously staining zone between the cell nuclei and those surfaces of the cells which are in relation to the epithelium of the neural plate. In an embryo of the early somite stage (Hamburger & Hamilton, stage 8 –) a small cavity has appeared in the centre of the somite, the cells are arranged about this and the dorsal, intensely staining zone is clearly visible and wider. Both Brachet (1940) and Gallera & Oprecht (1948) have observed this strong staining capacity of the somites. The latter, however, say that initially most stainable substance is about the central cavity.

Medullary plate and developing neural tube

As a result of the movements of gastrulation the developing neural epithelium comes to occupy an approximately oval area on the dorsal surface in the midline, the ventral surfaces of its cells being in direct contact with the now underlying notochord, cephalic mesenchyme, and somite mesoderm.

Comparison of the neural epithelial cells of early stages with those of a head fold embryo (Hamburger & Hamilton, stage 6) reveals certain changes in composition. In the latter the neural folds are well developed but the groove is not yet closed (Text-fig. 2). The epithelium of the groove and folds is composed of elongated cells. It is the most strongly pyroninophil region of the embryo (Plate 2, fig. D). Where it is continuous peripherally with the skin ectoderm the difference in pyronin staining between the two tissues becomes well marked as the neural tube closes. The superficial and middle cytoplasmic zones of the neural cells at this stage resemble the superficial zone of earlier stages and likewise contain a large amount of pyronin-staining substance but contain also bodies of various sizes which stain either little or not at all. The cytoplasmic zone beneath the nucleus and adjacent to the chorda-mesoderm contains a strikingly high concentration of pyroninophil substance, staining deeply and homogeneously (Text-fig. 2 and Plate 2, fig. D). This staining property of the basal cytoplasm of the neural cells is maximal at cranial levels where their development has progressed farthest. In a later stage embryo (Hamburger & Hamilton, stage 8 –) the basal concentration of basophil substance is maintained, particularly at cranial levels where closure of the neural tube is almost complete (Plate 2, fig. F). At more caudal levels, where the neural tube is in an earlier stage of its development, the neural epithelium already stains relatively strongly with pyronin, but the reaction of the basal cytoplasmic zone becomes progressively less distinctive in a cranio-caudal direction, the caudal cells grading into those of the primitive streak.

The appearance of the basal zone of the neural epithelium is in strong contrast with that of the yolk-laden basal zone of the earlier presumptive neural tissue.

Action of ribonuclease on the pyronin-reactive cytoplasmic substance

Treatment with ribonuclease destroyed the capacity of all the pyroninophil cell components described here for staining afterwards with pyronin (Plate 2, figs. D and E).

DISCUSSION

The work of Waddington (1932, 1933) has provided good evidence that induction of neural tissue by the axial chorda-mesoderm occurs in the chick. It is theoretically possible that the neural inductor may act in the two-layered stage of the embryo when the tissues concerned are adjacent zones of the epiblast, during invagination, or as the chorda-mesoderm becomes related to the ventral surface of the neural epithelium or during several or all of these stages.

It is reasonable to postulate a chemical basis for this and other inductions, in which case the stimulus from the living inductor may be visualized as involving a chain of reactions linked together by either (1) diffusion of a substance from one cell to another, or (2) contacts between reactive surface molecules at interfaces between cells. For the identification of ribose nucleic acid in these tissues use has been made of the ribonuclease test. This test cannot be regarded as specific for ribose nucleic acid but, on the basis of trial of a wide range of substances, may be taken to provide good evidence for its presence (Dubos & Thompson, 1938; Kunitz, 1940; Brachet & Shaver, 1948; McDonald, 1948; McDonald & Kaufmann, 1954).

The results confirm that there is a relatively high concentration of this basophil substance in the cytoplasm of the early epiblast cells (Brachet, 1940). Further, this study has shown that, at this time, the presumptive mesodermal and presumptive neural areas are not distinguishable from one another on the basis of their content of basophil substance or its distribution.

A visible differentiation of the two cell-groups under consideration from one another begins during invagination of mesoblast. Brachet described an accumulation of basophil substance in the cells at the lip of the blastopore and invaginating cells of amphibians and this was confirmed by Gallera & Oprecht (1948) for the primitive streak of the chick. This study shows that the cells invaginating through the primitive streak alter in shape and generally contain a high concentration of basophil substance throughout the cytoplasm by the stage at which they begin to disperse as mesoderm. Some cells, however, do not. Gallera &

Oprecht found that initially the more peripheral cells of the early mesoderm contain less staining substance, an observation difficult to explain since later the mesoderm generally becomes strongly staining. The present investigation revealed weakly staining cells interspersed throughout the invaginating mesoderm and early mesodermal tissue. This apparent heterogeneity of the streak and early mesoderm is no less difficult to explain. It is possible that the yolk-containing cells having little material stainable with pyronin are bottle cells with morphogenetic function, comparable with the blastoporal cells in amphibians described by Holtfreter (1943) and destined to disappear without differentiating further, but there is no real evidence for this here. After their invagination notochordal and mesodermal cells have generally a high content of basophil substance throughout their cytoplasm.

On the other hand, the cells of the neural epithelium, remaining on the surface, retain their concentration of basophil material in the supranuclear zone later. It appears that this substance is subsequently distributed in high concentration throughout the cytoplasm while yolk is breaking down and distinction between surface and deep zones is lost, and that it continues to increase in amount, a most striking concentration developing in the deep zone of the neural cells adjacent to the notochord and somite mesoderm. The intensity of staining here becomes much higher than in the underlying notochord. In this sense Brachet's observation of a high concentration at the interface between the two layers can be confirmed at this stage. Is there in this phase a transfer of ribose nucleic acid or some part of it from chorda-mesoderm to the bases of the neural cells? Except in one instance in which the cytoplasm of somite cells stained more intensely than that of adjacent neural cells the greatest intensity of staining was, at all levels and stages of differentiation examined, on the side of the neural cell cytoplasm. Thus generally no accumulation on the mesodermal side has been observed to precede the increase on the neural side of the interface. Although this by no means excludes the possibility of transfer, the appearances observed here could equally well be taken to indicate that, apart from any such transfer, there is a very active synthesis of the nucleic acid in the cytoplasm of the neural epithelium. The significance of this developing local concentration of basophil material in the neural cells remains to be determined.

SUMMARY

1. Basophil cytoplasmic substance of the cells concerned in neural induction in the chick was investigated by methyl green-pyronin staining and the action of ribonuclease. Comparison of a number of stages indicated that changes in both distribution and concentration occur.

2. Most of the basophil substance was in the dorsal zone of the cytoplasm in presumptive neural and presumptive mesodermal epiblast. In the subsequent differentiation of neural epithelium and chorda-mesoderm its concentration throughout the cytoplasm of both cell-groups increased considerably.

3. The highest concentrations observed developed in a sharply localized zone at the ventral poles of the cells of the neural groove in its early stages and in some somite cells.

4. The time relationships of concentration changes in adjacent cells did not indicate whether concentration of the substance at the base of the neural epithelium depends on transfer from other cells.

ACKNOWLEDGEMENTS

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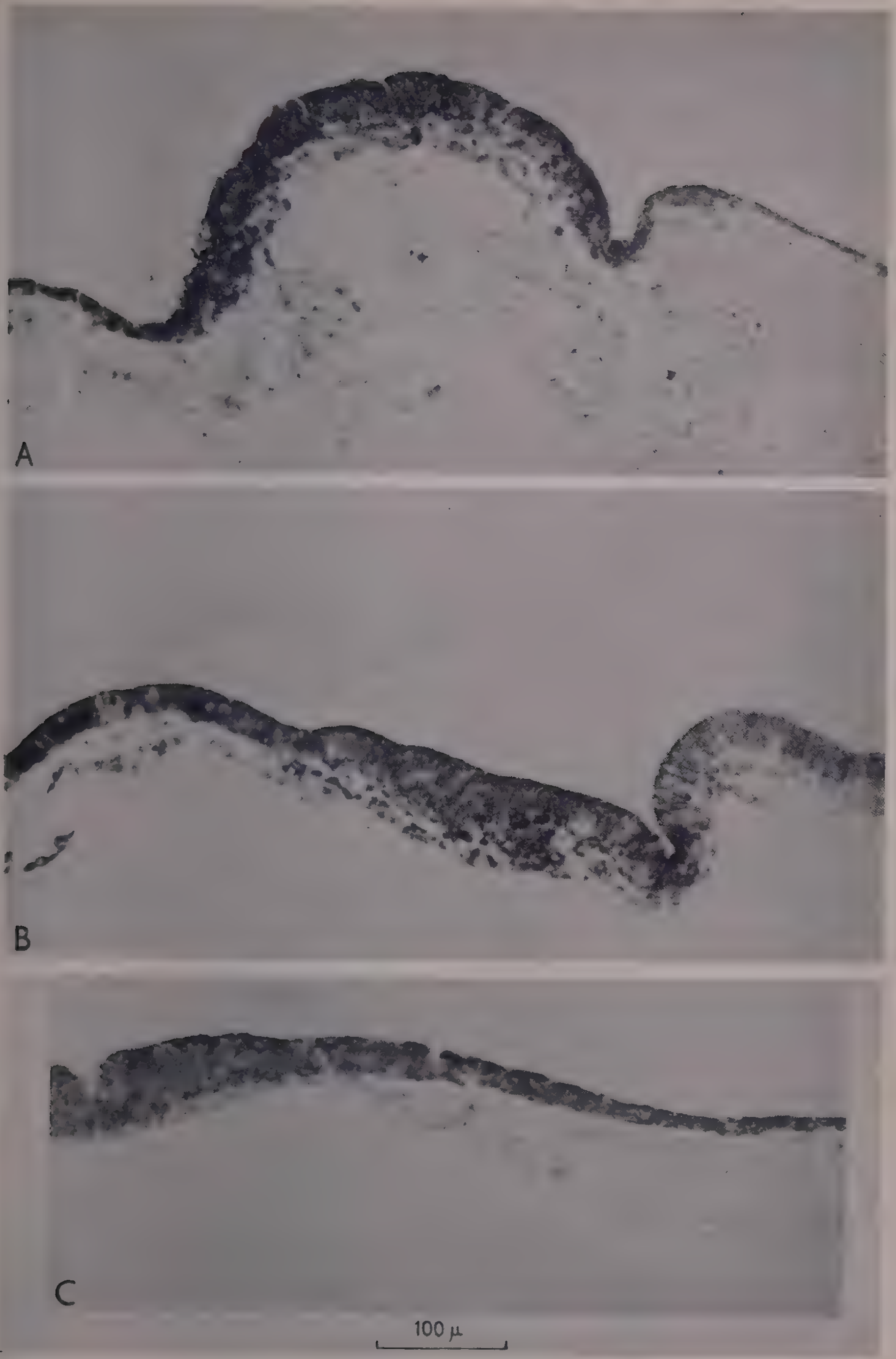

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Plate 1

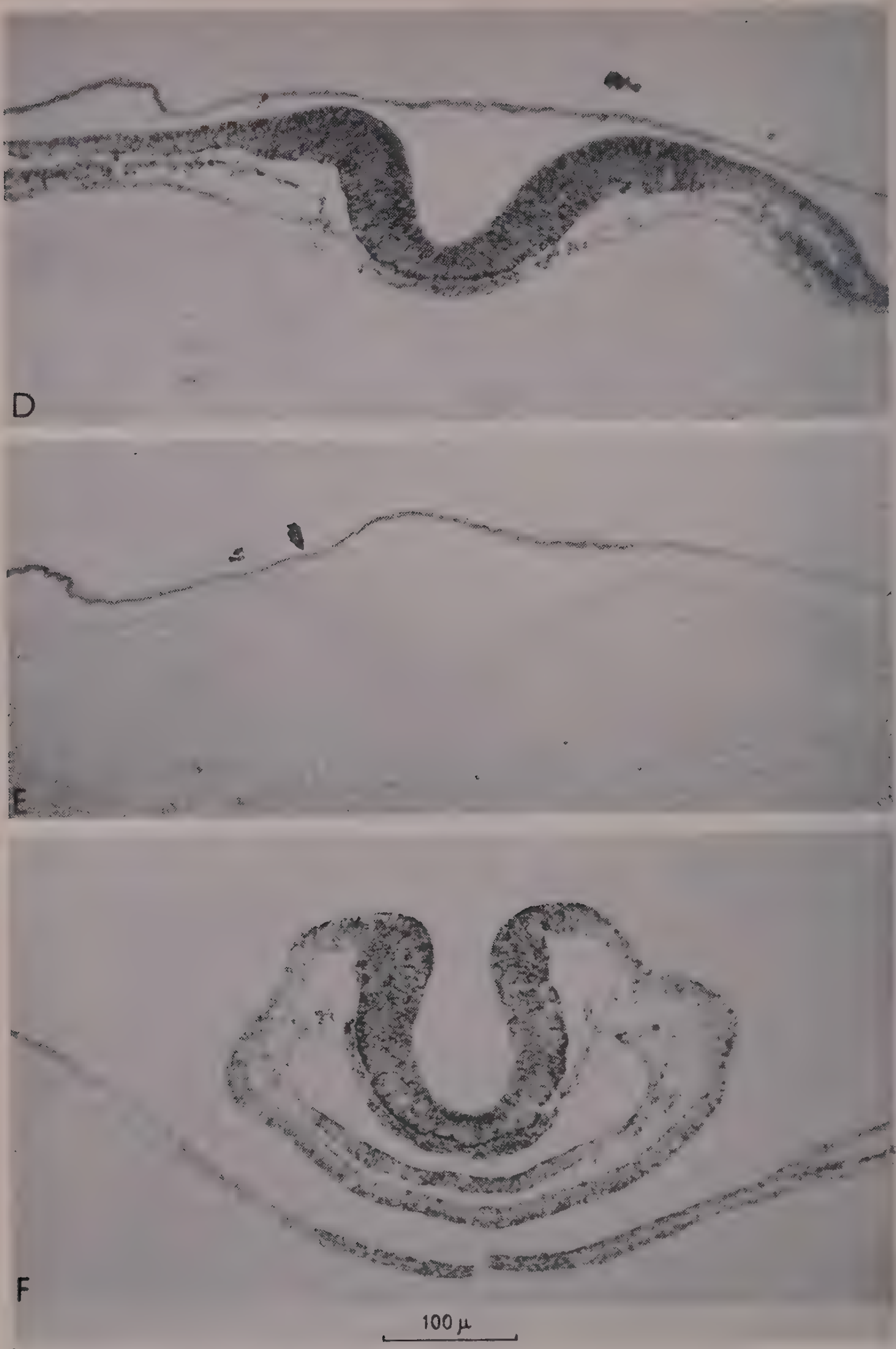

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Plate 2

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EXPLANATION OF PLATES

All photographs were taken with filters to demonstrate pyronin-staining material.

PLATE 1

FIG. A. Intermediate-streak embryo (stage 3+). Cross-section at the caudal end of the primitive streak. Distribution of basophil substances shown by methyl green-pyronin in ectoderm, primitive streak, and endoderm.

FIG. B. Intermediate-streak embryo (stage 3+). Cross-section at the cranial end of the primitive streak. Distribution of basophil substances shown by methyl green-pyronin in ectoderm, primitive streak, early mesoblast, and endoderm.

FIG. C. Head-process embryo (stage 5–). Cross-section at a caudal level in the primitive streak. Distribution of basophil substances shown by methyl green-pyronin. This shows a contrast between cells of the ectoderm and primitive streak and those of the endoderm beneath them.

PLATE 2

FIG. D. Head-fold embryo (stage 6). Cross-section through the neural tube. Distribution of basophil substances shown by methyl green-pyronin in neural epithelium, chorda-mesoderm, and endoderm. This shows the zone of high concentration at the bases of the neural cells.

FIG. E. Section, adjacent to that of fig. D, treated with ribonuclease and stained with methyl green-pyronin and photographed under the same conditions to show loss of pyroninophil material.

FIG. F. Early somite embryo (stage 8+). Cross-section in the head region. Distribution of basophil substances shown by methyl green-pyronin in neural tube and surface ectoderm, chord and mesoderm and foregut. This shows the zone of high concentration at the bases of the neural cells at a later stage.

(Manuscript received 31 : v : 56)

Functions of the Lymph Gland Cells during the Larval Period in *Drosophila*

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From the Institute of Animal Genetics, Edinburgh

WITH TWO PLATES

INTRODUCTION

A CONSIDERABLE body of information has already been accumulated in which a careful study of the morphological effects of a gene in *Drosophila* has been used to throw light on the epigenetic processes which bring about development. Much of the earlier work of this kind (e.g. Goldschmidt, Waddington) has dealt with mutant genes which produced abnormal adults. More recently a great deal of attention has been paid to the developmental effects of lethal genes which cause the death of the individual before the adult stage is reached. In a recent monograph on this category of genes, Hadorn (1951), who has been one of the most active workers in this field, lays considerable stress on what he calls the phase specificity of the lethals, that is, on the fact that individuals homozygous for a particular lethal usually die at some rather definitely defined stage of their life history. These phases at which death supervenes are perhaps more a matter of the clinical medicine of *Drosophila* than an aspect of its fundamental biology. There are undoubtedly certain times in the life history at which a particularly complicated and ticklish set of processes have to be carried out if the animal is to survive. They have been spoken of as epigenetic crises (Waddington, 1941). Developmental alterations at some earlier time may not have any very drastic effects on the viability of the organism until such a crisis arises, when they may give rise to gross abnormalities which bring about the animal's death. The fact that many lethals may be found to kill their bearers at the same phase (for instance at the metamorphosis from the larva into the prepupa) does not necessarily mean that the primary action of all these lethals has the same phase specificity. They may have begun to produce slight abnormalities at a much earlier stage. It is, indeed, by the investigation of these early stages of gene action that we may hope to throw light on the nature of the epigenetic crises and to discover the factors involved in them. One of the most important foundations for studies

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of this kind is a thorough knowledge of the development during the larval stages of the rudiments which will, after metamorphosis, give rise to the imaginal organs.

Shatoury (1955 *a, b, c*) has recently directed attention to the importance of the lymph glands in this connexion. Towards the end of both the second and third larval instars these glands become hypertrophied and eventually rupture, releasing into the haemocoele a number of freely dispersed cells. Histological evidence suggests that, at the end of the second instar, many of these cells accumulate around the imaginal buds and give rise to the imaginal mesoderm of the buds. This suggestion is confirmed by the fact that in one of the lethals (lethal no-differentiation) studied by Shatoury abnormalities among the early third instar lymph glands are followed by a pathological behaviour of this imaginal mesoderm. In another lethal (lethal malignant) a considerable hypertrophy of the glands gives rise to an excessive number of free cells which behave in a way reminiscent of a malignant tumour. The present paper deals with certain other groups of cells which appear in normal wild-type larvae, being apparently derived from the lymph glands and taking part in the differentiation during the larval period of the future imaginal rudiments. The cells with which we shall be concerned enter into the formation of the cephalic imaginal bud, the foregut (proventriculus), and the testes. A later paper will deal with the fate of similar cells in the mid-gut or stomach. The evidence that these cells are derived from the lymph glands comes partly from the fact that they appear in the various organs shortly after the release of cells from these glands, partly from the histological similarity between them and the cells within the glands, and partly from the fact that in their later development they show histological changes which parallel exactly changes also proceeding in the lymph glands themselves. Shatoury has described the lymph gland cells as falling into three main classes—rather large cells known as hexagons, and two smaller types known as spheroids and platelets. It is convenient to have some name for the whole group of cells which, emerging from the lymph glands, move around the body and settle in particular localities. We shall refer to them as oikocytes (derived from the Greek *oikizo*, 'to found a colony').

MATERIALS AND METHODS

The work to be described is based on the investigations of a wild-type OrK stock which had been inbred for a large number of generations and was obtained from the Institute of Animal Genetics, Edinburgh. Material was fixed at two-hourly intervals starting from 6 hours before the embryos hatch until the beginning of the pupal stage. Where accurate timing near the beginning of an instar was necessary, larvae were watched under the binocular and the time of moulting observed directly. Boiling Bouin was used as a fixative, and larvae were treated by Peterfi's method of double embedding. Prepupae and pupae were dissected out from their puparia and were treated by the amyl acetate technique.

Sections ranged from 2 to 5 μ and were stained with haematoxylin, considerable care being devoted to appropriate differentiation of the stain.

Oikocytes in the cephalic imaginal bud

At roughly 5 hours before the end of the second instar, the first and only defined pair of glands hypertrophies and releases its cells into the body cavity. It has already been pointed out (Shatoury, 1955*b*) that some of these cells form the imaginal disk mesoderm, whose possible influence on the process of differentiation of the buds, leading to a segmentation pattern characteristic of each, was discussed.

No differential selection of the migrating lymph gland cells by the various imaginal buds was observed in the previous investigations. However, the present study has shown that all the imaginal buds, except that portion of the cephalic complex which later differentiates into the optic disk, receive the same type of cells, which are either spheroids or platelets, or possibly a mixture of both. The mesoderm cells of the presumptive optic bud are of an entirely different type, as they seem to be made up of hexagons. In the other buds the hexagons come to lie either on the peripodial membrane or somewhere in its vicinity.

Till shortly before the end of the second instar the cephalic complex is similar in structure to any other imaginal bud, being composed of a simple epithelium flanking a peripodial cavity which is surrounded by a thin peripodial membrane (Plate 1, fig. A). As shown in the figure, the migrating mesoderm cells accumulate in two groups. One lies underneath the presumptive eye-disk portion and the other lies on the opposite side. Three hours before the beginning of the third instar the cephalic bud becomes differentiated into an antennal and eye segment. Fig. B shows that during this process of differentiation the hexagons lying on the peripodial membrane have divided the cephalic bud into an anterior cylindrical segment and a posterior oval one, representing the antennal and eye-bud respectively. Although these appearances have been overlooked, Chen (1929), Steinberg (1943), and others have already reported that the cephalic complex remains undifferentiated till about the end of the second instar.

At the beginning of the third instar the cephalic complex is distinctly differentiated into its two major components, which still remain connected by a thin membrane. The hexagons of the optic bud proliferate and the oval posterior side of the bud becomes concave (Plate 1, fig. C). A new cavity, which is occupied by the multiplying hexagons, eventually appears. The increase in the size of the optic bud is accompanied by a rapid increase in the number of the hexagons so that later stages (up to the 80-hour stage) show a considerable number of them.

At the 85-hour stage, and simultaneously with the melanization of the hexagons in the lymph glands (Shatoury, 1955 *a*, *b*), those in the optic bud behave similarly; this is shown in Plate 1, fig. D, where the optic hexagons have become deeply melanized. In older larvae they become vacuolated and finally disintegrate, and appear later as a homogeneous undifferentiated mass. Melanin deposition was shown by Shatoury to be a post-mortem event, perhaps brought about by a complement reaction.

Before melanin deposition has taken place the optic bud is seen to have become differentiated into two major segments, a head segment which metamorphoses to the adult cephalic integument, and an eye segment (fig. D). The latter in turn is sub-differentiated into three layers and it is in the uppermost of these that the precursors of the ommatidia first appear. Literature on the time of the onset and completion of this process is still in a confused state. Krafka (1924) and Chen (1929) reported that this differentiation begins about 24 hours before pupation. Medvedev (1935) mentioned that the ommatidia could be seen in whole mounts of optic disks of mature larvae. On the other hand, Bodenstein (1938) and Robertson (1936) claim that the ommatidia begin to differentiate 36 hours after puparium-formation. Enzmann & Haskins (1938) state that it starts 18 hours after egg-laying, but this has been proved wrong by Steinberg (1941). According to Pilkington (1942), the optic disk of a 4-day-old larva consists of undifferentiated cells several layers thick and apparently homogeneous. This contradicts Steinberg's report (1941) that the eye-disk begins to organize into clusters of four cells each at about 72 hours after hatching and that the clusters increase remarkably in both number and clarity in the 96-hour stage. The present investigations revealed that before the optic hexagons have disintegrated the eye segment of the optic bud has completely differentiated into ommatidia precursors. Further studies on the differentiation of these structures during the larval and post-larval stages are being carried out.

Oikocytes in the proventriculus

The hypertrophy of the lymph glands, followed by their rupture and the release of oikocytes into the haemocoele, occurs not only in the latter part of the second instar but at a similar stage in the first instar larva. Some of the cells released at this stage find their way into the proventriculus.

The oesophagus of the *Drosophila* larva is a narrow simple tube which passes between the brain hemispheres into the bulb-like proventriculus. In newly hatched larvae (Plate 2, fig. K), apart from the oesophageal component, the proventriculus is a compound structure composed of two walls. There is an outer layer of large cells of an intense affinity to basic dyes, which is regarded as an extension of the stomach. The inner layer is made up of faintly stained bow-like cells whose borders are poorly defined; the broad ends of the cells join, with the result that oval inter-cellular spaces occur. The nuclei are rectangular, and lie in the narrow central region of the cells with the major sides almost fused with the nearby cell-wall. The cytoplasm is reticular in texture, with thin strands connecting the nuclei with the cell-membrane. The internal structure of the nuclei is also important for our investigations. Nucleoli appear as rounded bodies which lie very close to the nuclear membrane. There are deeply stained granules of considerable size restricted to the periphery of the nuclei. This inner layer of cells is referred to here as the radial layer, and is usually regarded as an evagination of the oesophagus.

The oesophagus in the region where it joins the proventriculus is encircled by a ring of rather large cells whose function is uncertain; they were first described by Weismann and were named Weismann's cell chaplets or wreath cells. Investigations of these cells in first instar material shows that they are amoeboid, and are grouped in a loose sheet of cells embedded in a homogeneous matrix. It seems that this tissue receives a nerve-supply from the adjacent hypocerebral ganglion which lies somewhat at the anterior side of the proventriculus.

When the hypertrophied lymph glands release their cells into the body-cavity towards the end of the first instar, changes occur within the mass of wreath cells whereby they leave the matrix and wander about near by. As a result of this migratory movement the anterior end of the proventriculus becomes exposed in the body cavity. Lymph gland cells then accumulate in this region and penetrate between the wall of the oesophagus and the radial layer (Plate 2, fig. L). A narrow cylindrical cavity is thus formed between these two layers of cells. Figure L clearly illustrates that the migrating cells, while creeping through the newly formed cavity, become flat and tightly compressed.

In the selection of the hexagons and the rejection of the spheroids and platelets an important role seems to be played by the phagocytic activity of the wreath cells. There is other evidence that these cells have a scavenging function. For instance in larvae kept on food containing silver nitrate they become heavily charged with opaque particles. It seems possible that the reason why it is the hexagons rather than the other lymph gland cells which colonize the proventriculus is simply that the hexagons are the largest type of cell and therefore the most difficult for the wreath cells to ingest.

As soon as the hexagons establish themselves in the proventriculus (i.e. approximately 2 hours before the beginning of the second instar), the radial layer shows signs of gradual differentiation into two distinguishable regions. A few cells in the uppermost part aggregate, with the eventual loss of both the intercellular spaces and the original bow-like outline. They also differ from the rest of the cells in that the cytoplasm has lost its reticular consistency and has become more positive to basic dyes than that of the other radial cells, which remain entirely non-basophilic.

By the end of the second instar the upper cells have become sharply differentiated into a compact ring of a larger number of cells with a stronger basophilic reaction (the 'upper ring'). Nuclei are more positive to the stain than the cytoplasm and the individual cells have become spindle-shaped. The rest of the cells, which comprise the largest part of the radial layer, have also undergone changes, which in the main affect the nuclei; the basophilic granules have increased tremendously in number and instead of being restricted to the periphery, as in early first instar individuals, have become equally distributed throughout the whole of the nuclear material. The cytoplasm of these cells remains negative to haematoxylin.

It is not easy to decide whether the oikocytes which originated from late first instar lymph glands continue with differentiation processes during the third instar, or whether they die and are replaced by others derived from late second instar organs. However, microscopic examination suggests that they may undergo degenerative changes at the end of the second instar and that a fresh supply of hexagons from the hypertrophied second instar glands takes their place.

Although the hexagon component of the proventriculus is not particularly striking till the late phases of the second instar, it becomes a remarkable feature in the very early hours of the third. Plate 2, fig. M is from a 10-minute-old third instar larva and shows with clarity the exact location of the hexagons. It can also be seen that the upper ring of cells is proceeding to further differentiation; the cell number has increased and cell-walls have become more defined. Furthermore, the nuclei show a stronger basic reaction than the cytoplasm.

Successive stages in differentiation up to the 80-hour stage showed that the upper ring is undergoing progressive histological differentiation, with the nuclei still retaining a strong basophilic reaction. The remaining cells of the radial layer have also changed, in that the nuclei have become more organized and deeply stained. At the 85-hour stage the radial layer is clearly differentiated into the two types of cells. From now until the end of the third instar no further differentiation was observed in the radial layer.

After the differentiation of the radial layer has reached a state of completion, the hexagon cells of the proventriculus undergo changes of the same kind as those of the optic bud, that is, they become melanized (Plate 2, fig. N). This change is exactly simultaneous with the melanization of the optic bud hexagons and the similar cells remaining in the lymph glands. In later stages the hexagons of the proventriculus are displaced into the haemocoele (fig. O) where they disintegrate.

It has been believed by most previous investigators (Kowalevsky, 1887, on Muscids; Strasburger, 1932; Robertson, 1936, on *Drosophila*) that the imaginal fore-gut primordia is only represented by the upper ring. As a matter of fact, the remainder of the radial cells also take part in the formation of the imaginal organ.

At the beginning of metamorphosis the lower radial cells, which make up the greater part of the proventriculus, become attached by their inner ends to the wall of the oesophagus. At the white puparium stage the wall of the oesophagus becomes convoluted with the result that this organ contracts to less than half its original length. This process mechanically everts the radial layer of cells. Eversion of the two component primordia of the layer does not happen simultaneously but, owing to the fact that the lower cells have become attached to the oesophagus, they will be the first to be dragged forward (Plate 2, fig. P). Furthermore, in view of their characteristic pattern of distribution, it is natural to find that the everted cells come to lie side by side, forming a cylinder with their nuclei occupying its equator. Half-way through eversion, the cells of the main body of the radial layer have moved outside the proventriculus to constitute a thick ring (fig. Q). At this stage the upper ring has not been markedly affected by the process of eversion, but later it also is pulled out by the contracting oesophagus. When the process is completed both rings of cells form a compound waisted structure (fig. R) which is made up of two parts; an anterior region originating from the metamorphosis of the originally lower cells and a posterior region representing the everted upper ring of cells. Cellular proliferation of these 'imaginal' cells of the upper ring, as described by Strasburger and Robertson, has not been observed. This constricted structure will later metamorphose to the adult cardia.

Oikocytes in the testis

In the newly hatched larva of *Drosophila* the testis is undifferentiated and is only composed of spermatogonia. At the end of the first instar, which corresponds, according to the present investigation, to a few hours after the lymph glands have released their cells, the testis shows faint signs of differentiation to apical cells, spermatogonia, primary spermatocytes, and terminal cells. This remains the condition of the larval testis until shortly before pupation (Kerkis, 1933; Sonnenblick, 1941; Gloor, 1943; Geigy & Aboim, 1944).

In lethal malignant larvae, besides the principal malignant condition due to cells released from the lymph glands, Shatoury (1955*b*) has described a testis tumour tissue which invades the surrounding germinal layers, bringing about necrosis. In discussing the nature and origin of these cells, the hypothesis was suggested that they probably arise from cells of a pre-malignant type which lie at both ends of the gonads, which had been overlooked in previous investigations of its structure. A search for these hypothetical elements has now been made.

In larvae up to the 75-hour stage, preliminary efforts to identify them amongst the germ-tissue were unsuccessful. This was attributed to a possible equal stainability of these cells and the surrounding germ-cells. However, we have seen that the oikocytes in the proventriculus and optic disk exhibit a series of characteristic changes (pycnosis, melanization, or vacuolization) in the later stages of the third instar. It seemed possible that if similar cells occurred in the testis they would make their presence apparent at the same stage by processes of this kind. This was found to be the case. Plate 1, fig. I, which was taken from an 85-hour-old larva, shows two isolated groups of cells at the cephalic end of the testis in the neutral zone between the spermatogonia and the primary spermatocytes, which have become very striking in respect to their stronger affinity to basic stains, probably due to early symptoms of pycnotic degeneration. In 96-hour-old larvae (fig. J) these groups of cells have undergone lysis, so that in the prepupal testis their original place becomes represented by cavities. Similar cells (of undetermined number) with the same type of reaction were also easy to locate in the region between the posterior terminal cells and the primary spermatocytes.

These three groups of cells appeared consistently in the hundreds of preparations investigated.

The exact location of these cells having been accurately determined, histological examination of them in stages as early as late first instar were carried out. Plate 1, fig. H was taken from a stage slightly earlier than fig. I and represents one of these groups of new cells in the spermatogonial region before it shows the type of change just described. As shown, the cells are morphologically distinguishable from the surrounding ones. Figure G was obtained from a late first instar larva after the lymph glands have released their cells. It shows clearly two groups of three cells which are different from the rest of the spermatogonia not only from the morphological point of view but also from their cytochemical reaction towards basic stains. These groups of cells are located somewhat terminally, occupying both a cephalic and a caudal position. Prior to this stage lymph gland cells are found to accumulate in the region of the testis, probably suggesting that these cells are derived from migrating lymph gland material.

DISCUSSION

In the proventriculus, the optic imaginal buds, and the testis the investigations just described have revealed a very similar sequence of rather peculiar phenomena. At a relatively early stage in larval life, groups of cells, which seem to be identical with the hexagons of the lymph glands, make a sudden appearance. In the testis and proventriculus this happens during the late first instar, in the optic bud, during the latter half of the second instar. In both cases the cells appear just after the lymph glands have hypertrophied and released their contents into the body-cavity. Subsequent to the appearance of the cells in the organs, signs begin to be visible of the changes which bring the imaginal rudiments into the state of readiness in which they await the onset of metamorphosis. Thus in the testis there is the beginning of a differentiation of spermatogonia into early spermatocytes. In the proventriculus the upper ring of radial cells acquires a more solid consistency and a higher basophilia. In the optic bud the cells of the uppermost layer of the epithelium become arranged into the precursors of the ommatidia. There is a clear suggestion that the oikocytes act in some way as stimulators to further development.

One might perhaps be tempted to compare them to the organizers so well known in vertebrates, but it must be pointed out that the evidence suggests rather that they stimulate development to proceed farther along a course already laid down for it, rather than deciding a choice between alternative types of development as in the case of vertebrate organizers. If one were to seek an analogy for them among the entities known to play a part in early embryonic development it is perhaps in the well-known 'formation centre' (*Bildungszentrum*) described by Seidel in *Platycnemis* that the closest parallel might be found. It would, however, be premature to press such analogies too far, since it is clear that much remains to be learnt about the stimulating action which appears to be exerted by these cells. It seems technically impossible to investigate their action by the normal experimental embryological techniques of extirpation and grafting. The most hopeful line of attack would appear to be the discovery of lethal mutations which interfere with the migration of the hexagons to one or more of the organs involved. If the above hypothesis is correct, an organ to which the hexagons fail to arrive should not show any further histological differentiation during the second and third instars but should retain the character which it had during the first. Some evidence which supports this suggestion will be described in a later paper (Shatoury & Waddington, 1957).

The results of the transplantation experiments of several workers might appear to contradict the above hypothesis. For example, Steinberg (1943) transplanted early second instar cephalic buds into late second instar hosts and reported that the transplants differentiated normally. At the time of transplantation these buds were probably free of mesoderm. There is, however, no reason why mesoderm should not have been supplied to them by the lymph glands of the host larvae, and thus their behaviour provides no evidence that their differentiation is possible in the absence of the mesoderm.

It is interesting to note that immediately following the presumed inductive action of the hexagons, the tissue which reacts to them exhibits an increase in cytoplasmic basophilic granules. This reminds one of the phenomena described by Kedrowski (1937) and Brachet (cf. 1950) in the amphibian embryo. According to both these authors, these basophilic granules increase during histological differentiation and later decrease again as that process is completed. In *Drosophila* the imaginal primordia generally retain their basophilia until the beginning of metamorphosis, but it can be observed that the cytoplasmic granules disappear entirely while the pupal differentiation of the primordia is proceeding. Cytochemical tests are now being made to discover whether these transient granules show the characteristics of sulphhydryl ribonucleoproteins as do those described by Brachet.

One of the most remarkable features in the behaviour of the oikocytes is their disappearance towards the latter part of the third instar. They suffer a series of degenerative changes which may include pycnosis and vacuolization and often the deposition of melanin. These changes occur simultaneously with similar phenomena which affect the hexagons in the lymph glands themselves. Shatoury (1956), arguing from phenomena seen in certain lethal stocks, has suggested that the degeneration may be due to the action of antibodies formed in relation to the oikocytes which become antigenic at this stage. It must remain for future work to decide whether this hypothesis of autoantigenicity will eventually be found valid. In the meantime it provides a stimulating working hypothesis. Whatever the mechanism may be by which the oikocytes are caused to degenerate, it is surely noteworthy that this happens after the imaginal rudiments have completed the whole of that part of their differentiation which they carry out in the larval period and have reached a state of readiness to undergo the more profound changes which will occur on metamorphosis.

SUMMARY

1. In the latter half of the second instar the lymph glands of *Drosophila* larvae hypertrophy and release cells into the body-cavity, many of which move to the imaginal buds (Shatoury, 1955*a*). These cells have been referred to as oikocytes. The optic region of the cephalic disk becomes colonized by the large cells known as hexagons. During the first half of the third instar these increase considerably in number. At about the 85-hour stage, and simultaneously with similar changes in the cells still in the lymph glands, the oikocytes in the optic disk exhibit nuclear pycnosis and the deposition of melanin. Later the cells may become vacuolarized and disintegrate.

2. The precursors of the ommatidia can be seen in the optic disk before the oikocytes become melanized.

3. During the latter part of the first instar oikocytes are also released from the lymph glands, and hexagons accumulate around the end of the oesophagus where it enters the proventriculus. In the immediate neighbourhood of these oikocytes the radial layer of the proventriculus shows some cellular differentiation, forming an 'upper ring' of highly basophilic cells. It has usually been considered that this upper ring constitutes the primordia of the imaginal fore-gut, but it has been found that the lower radial cells will also contribute to this structure.

4. The oikocytes of the proventriculus suffer melanization and degeneration at the same time as those of the optic disk and the lymph gland.

5. In larvae aged between 85 and 96 hours two groups of degenerating cells can be found near the cephalic end of the testis and one group in the caudal end. These three groups of cells seem to be oikocytes, and can be traced back to the first instar, when they seem to enter the testis at the same time that the proventriculus is being colonized. Their appearance in the testis is followed by the first sign of the differentiation of spermatocytes.

6. The appearances suggest that the oikocytes, when they arrive at the testis, the proventriculus and the optic disk, play some part in stimulating those processes of differentiation which bring these organs into the condition from which they can undergo metamorphosis. The simultaneous degeneration of these cells at the end of the third instar would seem to indicate that this stimulus is no longer needed after that time. Shatoury (1956) has suggested that the mechanism which brings about this degeneration involves the formation of auto-antibodies.

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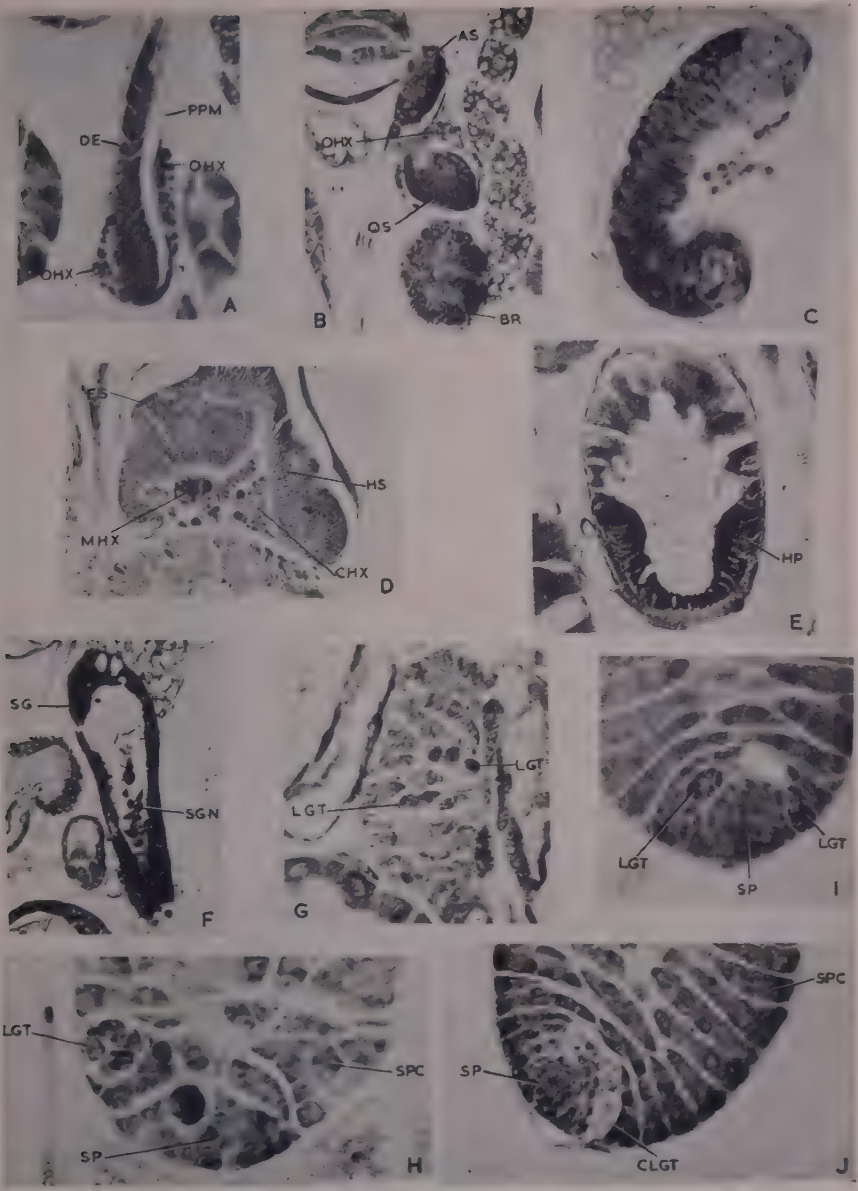
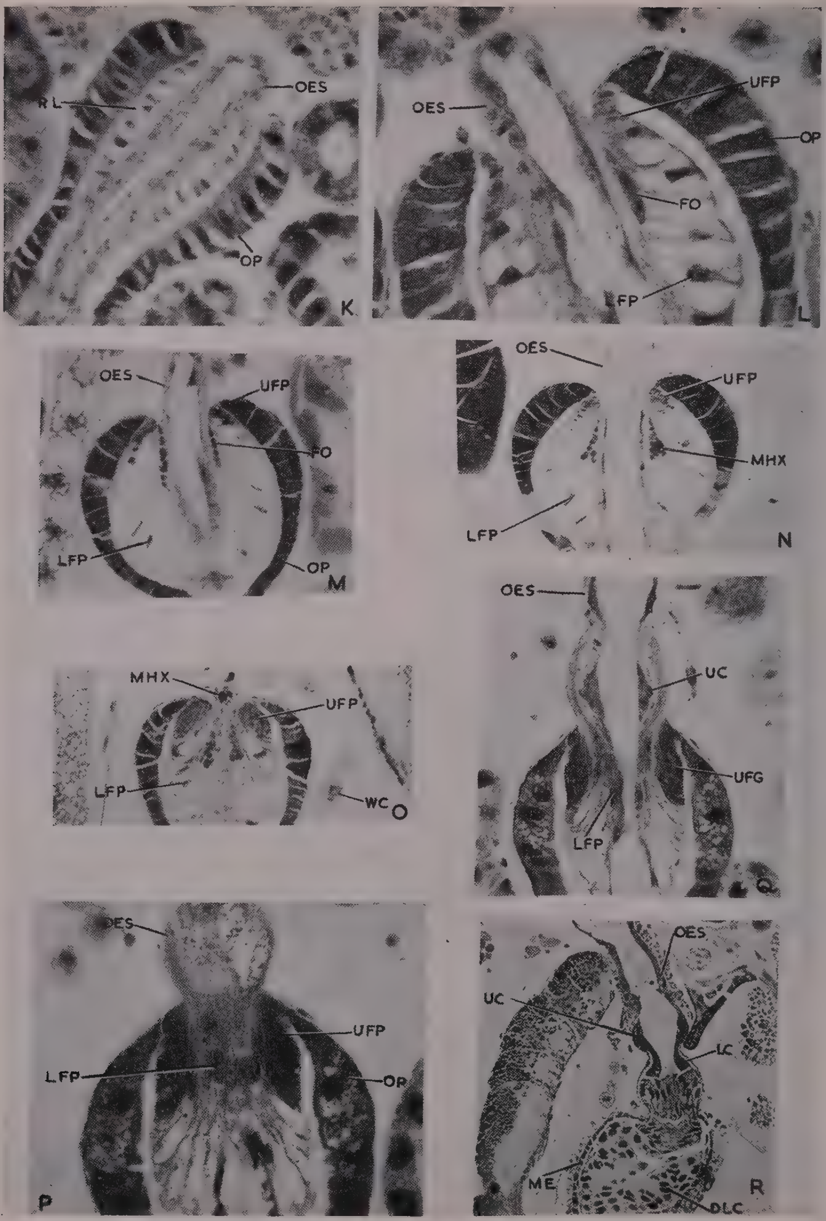
EXPLANATION OF PLATES

Abbreviations: AS, antennal segment; BR, brain; CHX, cytolysing hexagons; CLGT, cytolysing lymph gland cells; DE, epithelium of imaginal region; DLC, displaced larval gastric epithelium; ES, eye-segment of bud; FO, oikocyte of fore-gut; HP, primordium of imaginal hind-gut; HS, head



H. H. EL SHATOURY *and* C. H. WADDINGTON

Plate 1



H. H. EL SHATOURY *and* C. H. WADDINGTON

Plate 2

segment; LC, lower half of cardia; LFP, lower cells of fore-gut primordium; LGT, lymph gland cells; ME, imaginal mid-gut epithelium; MHX, melanized hexagon; OES, oesophagus; OHX, 'Hexagons' of optic segment; OP, outer layer; OS, optic segment; PPM, peripodial membrane; RL, radial layer; SG, salivary gland; SGN, cellular masses resulting from hypertrophy of salivary gland imaginal primordium; SP, spermatogonia; SPC, primary spermatocytes; UC, upper half of imaginal cardia; UFP, upper ring of cells; WC, 'Wreath cell' of Weismann's cell chaplet.

PLATE 1

FIG. A. Late second instar cephalic bud. × 240.

FIG. B. Very late second instar cephalic bud, showing the differentiation of the antennal segment (AS) and optic segment (OS). × 240.

FIG. C. Five-hour-old third instar bud, showing oikocytes underlying the optic region. × 440.

FIG. D. Section of brain and optic bud in late third instar, showing melanization of hexagons. × 400.

FIG. E. Section of hind-gut, mid third instar.

FIG. F. Late second instar, section through anterior end of salivary gland. × 180.

FIG. G. Late first instar testis. Note that the bulk of the testis shows no differentiation into spermatogonia and spermatocytes. × 130.

FIG. H. Third instar, 80 hours; cephalic region of testis. × 400.

FIG. I. Eighty-five-hour testis, showing two groups of pycnotic lymph gland cells near cephalic end. × 220.

FIG. J. Ninety-six-hour-old testis, showing cytolysing lymph gland cells (CLGT). × 240.

PLATE 2

FIG. K. Proventriculus of newly hatched larva. × 220.

FIG. L. Late first instar proventriculus. × 440.

FIG. M. Ten-minute-old third instar; note large number of fore-gut oikocytes. × 220.

FIG. N. Late third instar. × 190.

FIG. O. Late third instar. Displacement of melanized hexagons into body-cavity. × 170.

FIG. P. Prepupa. Early stage in eversion of fore-gut primordium. × 330.

FIG. Q. Later stage in eversion of primordium. × 330.

FIG. R. Completion of eversion of fore-gut primordium. × *c.* 130.

(Manuscript received 27 : vi : 56)

Development of the Intestinal Tract during the Larval Period of *Drosophila*

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WITH ONE PLATE

INTRODUCTION

THIS paper is concerned with certain aspects of the development of the mid-gut (stomach), hind-gut, and salivary glands of *Drosophila* larvae. Attention will be particularly concentrated on two types of phenomenon, firstly, on periodic processes of hypertrophy and regression which affect certain of the larval tissues comprising these organs, and, secondly, on the development of groups of imaginal cells which, at metamorphosis, produce the tissues from which the adult organs are built up. There is evidence that in the control of both these processes an important part is played by the lymph glands. This evidence is derived primarily from the study of certain lethal mutant types which will be described in a later communication (Shatoury & Waddington, 1957*b*). In these lethals it is found that abnormality of the lymph glands is associated with and appears to be the cause of excessive hypertrophy of the larval cells composing the gut. Certain other observations from the same lethals suggest that the cells which make up the imaginal rudiments are originally derived from the lymph glands, belonging to the type which has been designated as 'oikocytes' (Shatoury & Waddington, 1957*a*), i.e. cells which are given out from the lymph glands and which settle as colonies in certain other regions of the larval body. It will therefore be necessary to indicate the temporal relations between the processes which occur in the intestinal tract and phenomena which can be seen in the lymph glands.

The material on which the investigation was based consists for the most part of a large series of sectioned specimens which were fixed at two hourly intervals starting from 6 hours before hatching until the beginning of pupation. This was the same series as was used in a previous communication (Shatoury & Waddington, 1957*a*) where the histological methods have been briefly described. Certain supplementary preparations have been made to check special points.

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RESULTS

Changes in the lymph glands during the first and second instars

During the latter half of each of the three larval instars the lymph glands hypertrophy. The membrane surrounding them ruptures, the cells are released into the haemocoele. The events at the end of the third instar have been fully described by Shatoury (1955*b*) and we shall not be further concerned with them here. It will be necessary, however, to give some data about the processes in the first instar and to add to the descriptions of the second instar events which were given by Shatoury (1955*a*).

During the first instar only the first (most anterior) pair of lymph glands is developed, although the remaining pairs of glands probably occur as minute primordia. By the seventeenth hour in the first instar the glands are quite large in size. Very shortly after this the glands rupture and cells are released into the haemocoele. The majority of these move caudally and become attached to the hypodermis of the last abdominal segment where they later disintegrate. Probably some of them take part in a transitory formation of a ventral hypodermal organ since this shows some sign of hypertrophy at this stage. Others seem to move into the proventriculus and the testes as described previously (Shatoury & Waddington, 1957*a*). Very soon after the rupture of the glands regenerative processes occur so that by the end of the instar the first pair of glands is reconstituted. The process of regeneration at this stage has not been exactly studied. There is no enlargement of the primordia of the other glands at this period.

During the second half of the second larval instar the first pair of lymph glands again hypertrophies, ruptures, and releases cells into the haemocoele. The sequence of events can be described in the following stages:

(1) Hypertrophy of the first pair of glands with cellular proliferation, breakdown of the confining membrane, and release of cells into the body-cavity.

(2) Growth of the primordia of the other four or five pairs of glands which make up the complete set of glands in third instar individuals. At this stage the first pair of glands still contain a considerable number of cells but these show nuclear degeneration and vacuolated cytoplasm.

(3) Disintegration of the cells of the first pair of lymph glands; the glands in section appear to contain mainly a featureless coagulum containing fragments of degenerating cells.

(4) Regeneration of the first pair of lymph glands. This process occurs at just about the time when the larvae shed their cuticle to develop into the third instar. Plate, fig. A, is taken from a 15-minute-old third instar larva and shows the first and second pairs of glands. In the former the clear areas are part of the coagulum which represents the disintegration product of the cells of the earlier organ, while the cells are part of the complement which will fill the regenerated gland during the third instar. The source of these cells is probably to be found in the small primordium which lies just posterior to the gland. This is similar in

structure to the primordia which can be found earlier in the second instar and which develop into the more posterior glands. The regeneration of the first pair of glands, although underway at the time when the larvae shed their cuticle, is only completed about an hour later.

According to Poulson (1945) the first pair of lymph glands is actually detectable in embryos from 11–12 hours old and is derived from the embryonic dorsolateral mesoderm just posterior to the lobes of the brain. Since these glands exhibit periodic hypertrophy and regression during each of the larval instars, it was interesting to investigate whether they also do so during the embryonic period. In point of fact a process of this kind seems to occur at about the 20-hour stage in the embryo. The cells released at this time probably give rise to the imaginal primordia of the salivary glands and hind-gut and the so-called basement cells of the stomach which are described below.

Periodic hypertrophy and regression in the mid-gut, salivary glands and hind-gut

During the early part of larval life up to the middle of the second instar the mid-gut (stomach) is composed of a thick epithelium of large cells furnished with polytene nuclei and bounded externally by a basement membrane. Beneath the large epithelial cells, in immediate contact with the basement membrane, there may be found a small number of more deeply staining cells which are quite small in size. These will be referred to as the 'basement cells'. It will be seen later that the primordia of the imaginal mid-gut form around these basement cells but it seems that the latter do not actually enter into the make-up of the final adult organs. The basement cells can be detected in newly hatched larvae but could not be found in preparations of late embryonic stages. It seems rather probable that they, and the imaginal primordia of the salivary glands and hind-gut which will shortly be mentioned, are derived from cells released from the lymph glands at the end of the embryonic period.

At the anterior end of each salivary gland there is a small group of cells which in the pupal period proliferate and reconstruct the histolysed larval glands during metamorphosis. Again in the hind-gut there is a half ring of cells (cf. Shatoury & Waddington, 1957*a*, Fig. E) lying just caudal to the entrance to the Malpighian tubules which renews the histolysed hind-gut epithelium and constitutes the imaginal rudiment. Both the salivary and hind-gut imaginal primordia appear in the hatching embryo but it is only in later larval stages that their differential stainability, due to the increase in their basophilia, distinguishes them clearly from the adjacent tissues.

At the time in the second instar when the first pair of lymph glands hypertrophies and releases cells into the body-cavity, the larvae are found either to leave the food completely or to remain in the food mass without showing any movement of the musculo-pharyngeal apparatus. Histological examination of specimens at this stage shows that the alimentary canal has become occluded by hypertrophy of the stomach epithelium. The most obvious expression of this hypertrophy is an expansion of the ends of the cells which line the lumen of the stomach. These enlarge in size and become differentiated into a main rounded part and a long basal extension which joins the bulk of the epithelium (Plate, fig. B). The rounded lumps of cytoplasm are rich in basophilic granules but few of them appear to be nucleated. They are eventually thrown off into the gut lumen and the proliferation of these anuclear 'cells' continues until the gut lumen is full of them (Plate, fig. C).

This hypertrophy of the wall of the stomach continues so long as the first pair of lymph glands is releasing cells into the body-cavity. As soon as the rudiments of the more posterior lymph glands enlarge the hypertrophy ceases and the larvae very quickly return to the food. The cytoplasmic masses in the gut-cavity soon break down so that the lumen is filled with a more or less homogeneous cytolysed mass of tissue which still has a strong affinity for basic dyes. Later this material appears as a layer surrounding the food material which is again passing down the centre of the gut-lumen (Plate, fig. D). Most of this cytolysed material seems to be absorbed again by the cells of the gastric epithelium, which increase in size, the part of the cell towards the lumen now being distinguished by a low stainability in haematoxylin (Plate, fig. E). Some of it remains lining the walls of the stomach and then completely loses its affinity for stain.

In the same stage in the second instar at which the large cells of the gastric epithelium hypertrophy, there is a considerable multiplication of the basement cells. These elements are oval in shape and show strongly hyperchromatic nuclei which lie rather asymmetrically to one side of the cell. The staining reaction of their cytoplasm is generally of the same intensity as that of the cytoplasm of the overlying epithelium (Plate, fig. F). In earlier stages only a comparatively few scattered cells are present but at the end of their multiplication they constitute a loose layer underlying the whole of the epithelium. They are confined to the stomach and have not been found in connexion with either the oesophageal or hind-intestinal epithelia. There are two hypotheses which might account for the situation described above. It might be that a common factor has stimulated both the gastric epithelium to proliferate to form the basophilic, anucleate cells, and the underlying cells to produce indifferent ones like themselves. The alternative hypothesis is that the causative factor only stimulated the underlying layer of cells to multiply, and this has reacted on the epithelial cells, resulting in cellular hypertrophy of a different type.

Simultaneously with the hypertrophy of the stomach cells, the half ring of imaginal hind-gut primordia, whose cells metamorphose to the adult hind-intestine, behaves in a similar way. They multiply rapidly, discharging their proliferations in the lumen of the hind-intestine. Cell hypertrophy ceases also at the time when the new lymph glands appear. Cytolysis follows, and the resulting substance is completely absorbed by the hind-gut epithelium.

The third tissue which exhibits similar tendencies at this time is the imaginal salivary gland primordium, which is composed of a ring of condensed spindlelike cells. The latter proliferate in the cavity of the salivary glands, resulting in cellular masses of a type different from the dividing mother cells. These are illustrated in Fig. F of Shatoury & Waddington (1957*a*), where the salivary gland hypertrophy has given rise to a group of large non-nucleated masses of a strong affinity to basic stains. Again, they later cytolyse, eventually being absorbed by the larval salivary gland cells which, unlike those of the gastric epithelium, do not exhibit any tendency to proliferation or hypertrophy.

We thus see that changes in the first pair of lymph glands are followed by three remarkable events which take place simultaneously. These involve hypertrophy or proliferation of the imaginal salivary gland primordia, the hind-gut primordia and the two components of the wall of the stomach. These growths regress at the time of the development of the rest of the set of the lymph glands as the third instar begins.

These processes have been described in some detail in the form in which they are seen in the second instar. In the first instar, at the time when the lymph glands hypertrophy and release cells into the haemocoele, the mid-gut, hind-gut, and salivary glands exhibit the same type of changes as those just described, though they are not so clear-cut or easy to detect.

Later differentiation of the imaginal mid-gut primordia

After the second-instar hypertrophy of the stomach has regressed, some lymph-gland cells, which are very similar in structure to third instar normal platelets, penetrate the basement membrane of the stomach and lie underneath the epithelial layer. In fact, it was found that the migrating cells rest upon the basement cells referred to above. Therefore, at this stage of development, which is just a few hours before the larvae moult to the third instar, the wall of the stomach is seen to consist of cells of three different types—large epithelial cells, some of which are sheltering a group of two cells, one lying over the other, these being, from outside to inside, a basement cell, and a lymph-gland cell or oikocyte. It is these last which differentiate into the imaginal mid-gut primordia, and the different stages in the differentiation process can be summarized as follows.

The solitary lymph-gland cells multiply and each gives rise to a group of a variable number of rather rounded sister cells (Plate, figs. G, H). In some preparations late anaphase chromosomes were frequent. Cell-division is completed by roughly the 75-hour stage, at which time the cells comprising each group are at least morphologically identical. At the 80-hour stage the outermost cells of these primordial groups change in shape and are drawn out into several processes. The basal extensions of the cells which undergo these changes meet and sometimes overlap, and the upward processes grow and reach the top of the surrounded cone of primordial cells. In later stages of differentiation, an imaginal primordium appears to consist of a group of four or five condensed cells tightly surrounded in all directions by the extended processes of the peripheral cells (Plate, fig. I). As illustrated by the figure, the surrounding processes elongate considerably as the primordia increase in size, so that they appear thin and fibre-like.

The growth and differentiation of the mid-gut primordial cells is accompanied by some multiplication of the basement cells over which the oikocytes have originally been seated. However, the basement cells multiply at a low rate, and by the 80-hour stage they appear as patches of two or three cells which fit snugly into the overlying groups of oikocytes (Plate, fig. J). The larval differentiation of the mid-gut primordia is almost completed at this stage, by which time the imaginal buds and the different components of the eye disk have also completed their pre-metamorphic differentiation.

At the 85-hour stage, when the oikocytes in the lymph glands and underneath the eye disks become melanized (Shatoury & Waddington, 1957*a*), the basement cells connected with the imaginal mid-gut primordia also undergo changes of the same type, in which the nuclei break down and the cells disintegrate. These cytolysed cells appear in 96-hour-old larvae as small granules lying against the basement membrane, and give the appearance of a muscular layer.

At the beginning of the white-puparium stage the stomach epithelium contracts to less than a quarter of its original length. This causes the primordial nests, which were rather widely scattered, to lie much closer to each other. Later they unfold, and the enclosed oikocytes, which have become triangular, spread over the cytolysed layer of the basement cells. A new epithelium composed of loose cells with inter-spaces is thus formed. In 3-hour-old prepupae, the larval cells are completely displaced into the gut-lumen, where they disintegrate and show pycnotic nuclei. The imaginal epithelium is reconstructed solely from the oikocytes from the central part of each primordium; the more superficial cells are discharged into the gut-lumen, where they elongate further and form a continuous layer around the histolysed larval cells (Plate, fig. K). Further contraction of the imaginal mid-gut leads to the disappearance of the intercellular spaces, and the stomach appears as a continuous epithelium.

DISCUSSION

During the development of a holometabolus insect through the larval instars, periodic phenomena of cell hypertrophy and proliferation occur in a number of tissues. They are particularly marked in the hypodermis in connexion with the formation of the new cuticle. They are not so well known in the internal organs. The peculiar phenomena which have been described here as occurring in the imaginal salivary gland primordia, the hind-gut primordia, and the components of the wall of the stomach in *Drosophila* might well have been overlooked were it not for the exaggerated form in which they appear in certain mutant stocks which will be described later. A discussion of their significance may well be postponed until the observations on the mutant stocks can also be taken into consideration.

It has been shown (Shatoury, 1955*a*; Shatoury & Waddington, 1957*a*) that in several larval organs such as the imaginal disks and the proventriculus, cells which are apparently derived from the lymph glands make a sudden appearance at some definite stage in development and appear to play a part in bringing the imaginal rudiments into a state at which they become ready for the processes of metamorphosis. In the mid-gut there seem to be two such periods of colonization by lymph-gland cells. The basement cells are present in the newly hatched larva and it seems likely that they are derived from the lymph glands during the last hours of the embryonic existence. A second colonization takes place during the second instar. In later development the basement cells and the oikocytes arriving in the second instar behave rather differently. The latter multiply during the third instar to form small groups of cells which constitute the primordia from which the adult mid-gut will be derived. At the time of metamorphosis these cells show no tendencies for degeneration but, on the contrary, multiply rapidly to form the adult organ. The basement cells, on the other hand, undergo degeneration towards the end of the third larval instar. The histological changes they exhibit are somewhat similar to those shown by the large oikocytes (hexagons) in the lymph glands, proventriculus, optic buds, and in the posterior end of the haemocoele. But in normal development, at least, the basement cells of the mid-gut do not suffer from the deposition of melanin which is found in the other types. Again, however, certain lethal mutants reveal the affinities of these cells and show that they also can undergo melanization (Shatoury & Waddington, 1957*b*).

SUMMARY

1. A few hours before the end of the second larval instar the first pair of lymph glands hypertrophies, then releases cells into the haemocoele. This is quickly followed by the growth of the primordia of the more posterior pairs of lymph glands. The cellular contents of the first pair of glands then disintegrates nearly completely and the glands are regenerated from small primordia lying at their posterior end. A similar but less well-marked process takes place at the end of the first larval instar.

2. At the time of the hypertrophy of the first pair of lymph glands in the second larval instar (and to a lesser extent also in the first instar) the main epithelium of the mid-gut hypertrophies, giving off a large number of lumps of cytoplasm, mostly without nuclei, into the lumen of the gut which becomes temporarily filled up with them. When the more posterior lymph glands develop this hypertrophy ceases, the cytoplasmic masses cytolyse and are largely resorbed by the mid-gut epithelium.

3. Rather similar processes of hypertrophy occur in the imaginal rudiment at the anterior end of each salivary gland and in the imaginal rudiment of the hind-gut which lies just caudal to the entrance of the Malpighian tubules.

4. Lying beneath the main epithelium of the mid-gut in the newly hatched

larva are scattered small cells referred to as basement cells. It seems likely that these are derived from the lymph glands during the end of the embryonic period.

5. Towards the end of the second larval instar the basement cells are joined by further lymph-gland cells. During the third larval instar these multiply, forming small imaginal primordia each consisting of a nest of deeply basophilic cells, the more peripheral cells throwing out long processes which extend round and enclose the others.

6. Towards the end of the third larval instar, when the lymph gland hexagons become melanized and degenerate, the basement cells also suffer degeneration though not melanization. At the time of puparium formation the central cells of the imaginal primordia proliferate to form the wall of the imaginal mid-gut. The peripheral cells of the imaginal primordia are thrown off into the gut-lumen where they form a continuous layer around the degenerating main larval epithelium.

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EXPLANATION OF PLATE

Abbreviations: BM, basement membrane; BZ, basal group of cells from which first gland is repopulated; CGN, cytolysing gastric proliferations; EMGP, cells derived from the periphery of the imaginal primordia which are now forming a membrane round the displaced larval gastric epithelium; GE, main gastric epithelium; HT, heart; ME, imaginal mid-gut epithelium; MGP, mid-gut primordia; MO, 'basement cells'; NGE, reconstituted part of gastric epithelial cells; PM, peritrophic membrane; RLG, regenerating first gland; SDL, second gland enlarging; ST, structureless stroma left by degenerated cells of first gland; TC, terminal cytoplasmic bleb derived from hypertrophied cells.

FIG. A. Very late second instar. Regeneration of first pair of lymph glands and growth of second pair. ×330.

FIG. B. Late second instar. Hypertrophy of gastric epithelium. ×670.

FIG. C. Late second instar. Stomach filled with cytoplasmic masses derived from hypertrophied epithelium. ×370.

FIG. D. Cytolysis of cytoplasmic masses filling stomach. ×170.

FIG. E. Absorption of proliferated masses by gastric epithelium and formation of 'peritrophic membrane'. ×150.

FIG. F. Early third instar, wall of stomach. ×730.
FIG. G. Early third instar, beginning of the formation of imaginal mid-gut primordium. ×1,000.
FIG. H. Third instar. Multiplication of cells of imaginal mid-gut primordium. ×670.
FIG. I. Fairly late third instar. Fully developed imaginal mid-gut primordium. ×1,200.
FIG. J. Late third instar. Cytolysis of 'basement cells'. ×1,200.
FIG. K. Prepupa. Metamorphosis of imaginal mid-gut. ×400.

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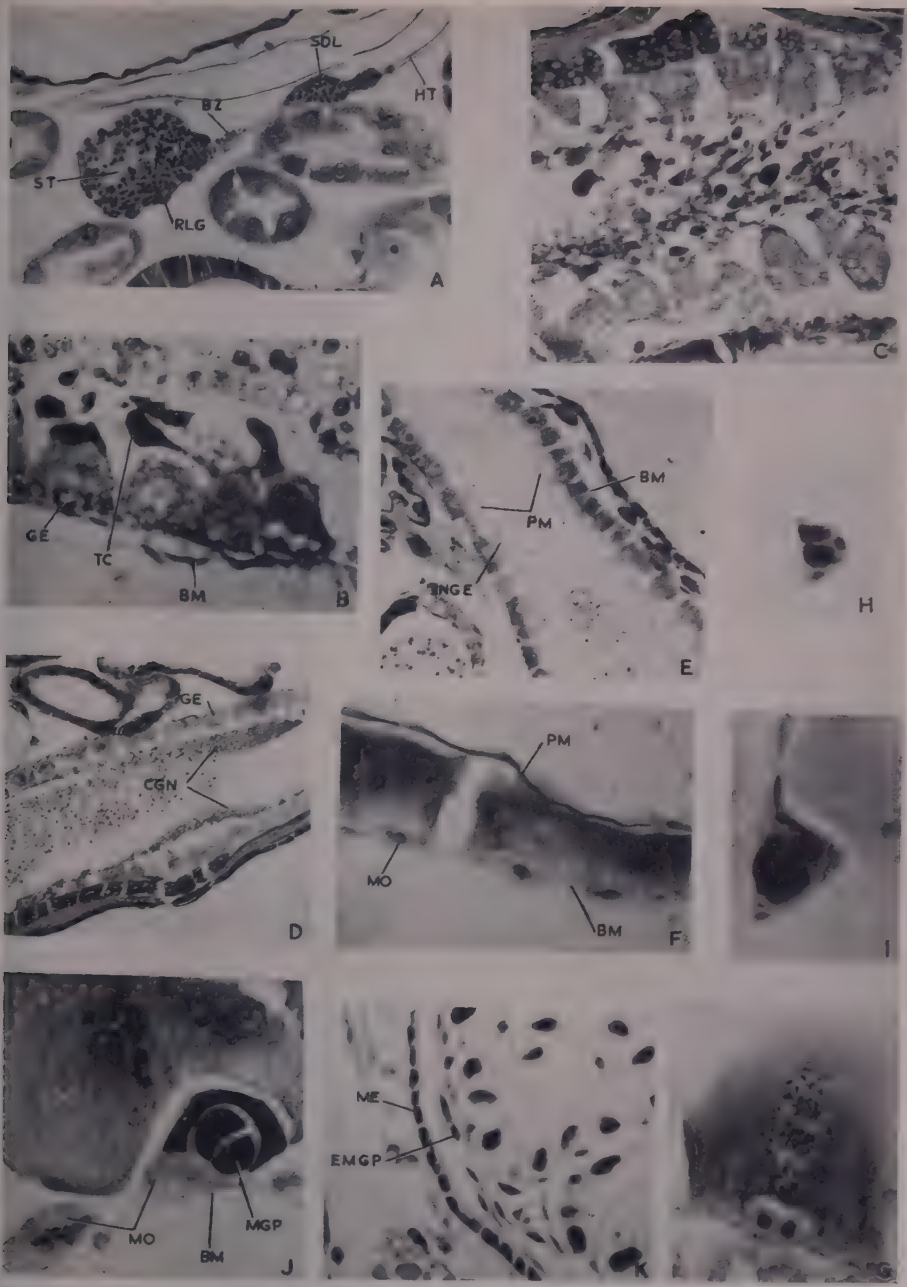

H. H. EL SHATOURY *and* C. H. WADDINGTON

Plate

The Development of Gastric Tumours in *Drosophila* Larvae

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WITH TWO PLATES

INTRODUCTION

It has been shown that in the normal development of wild-type *Drosophila* larvae, a process of hypertrophy or proliferation leading to the formation of groups of non-nucleated cellular masses occurs in the mid-gut (stomach) and at the imaginal primordia of the hind-gut and the salivary glands in both the first and second instars (Shatoury & Waddington, 1957*b*). The process takes place just at the time when the lymph glands hypertrophy and release cells into the body-cavity and the excessive growths of the organs of the alimentary tract regress and are resorbed as soon as the lymph glands become regenerated. The appearances strongly suggest that there is a causal connexion between the proliferation of the lymph glands cells and the hypertrophy which occurs in the gut and salivary glands. The present paper deals with development in two types of lethal mutant in which abnormalities of the lymph glands are followed by excessive hypertrophy of the gut, the growths of which attain such large proportions that they may truly be considered as neoplasms or tumours.

Most of the paper is concerned with a sex-linked lethal which has been given the name 'lethal no-imaginal buds' (l-nib). Some further details are also given concerned 'lethal malignant', which has been described previously (Shatoury, 1955). These lethals were carried in stocks balanced against the Muller-5 chromosome. The lethal male larvae were recognized by their yellow malpighian tubules and large gonads. In the histological preparation double embedding with celloidin was used.

RESULTS

Lethal no-imaginal buds

When this stock was first looked at in February 1953, only the late third instar larvae were examined. The larvae were rather smaller than normal and the testes

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were unusually small, which made it somewhat difficult to distinguish the male from female larvae in the cultures. There was little sign of abnormal lethality amongst the larvae and puparium formation was not delayed for more than about a day. The lethal individuals died as young prepupae. The most noticeable morphological fact about them was that the imaginal buds were either completely absent or exceedingly small.

Since that time the stock has been carried on, usually by females which were mated to their brothers. Occasionally, owing to shortage of males in the cultures, Muller-5 males from other stocks have been used to carry on the culture. When the stock was re-examined in January 1955 it was found that the lethal larvae showed a rather different phenotype, presumably as a result of this outcrossing. The first instar larvae and the majority of the second are morphologically normal. Lethal males leave the food immediately after they moult to the third instar and crawl about in the culture dish. They later develop a macroscopic black body in the region of the hind-gut imaginal primordium. Larval life ranges from 3 to sometimes 5 days after hatching. Puparia are never formed and larvae die as dwarf third instar larvae, which become translucid and swollen, with twisted broken trachae (Plate 1, fig. B).

In sectioned material the first instar larvae are anatomically normal. The first pair of lymph glands hypertrophies at the end of the first instar and some of its cells form the oikocyte content of the proventriculus, while others accumulate in the testes. The hypertrophy of the first pair of glands is followed, in the usual way, by the overgrowth of the stomach epithelium and of the imaginal primordia of the salivary gland and hind-gut.

Late first instar larvae, in which the first pair of glands would normally have regenerated, may be classified into three main groups:

(1) The majority of larvae showed the regenerated glands slightly smaller than normal.

(2) Some specimens showed moderately sized glands.

(3) In a few individuals the glands were still smaller.

In the first group there appear to be some abnormalities in the oikocytes which migrate to the testis (Shatoury & Waddington, 1957*a*). In the second group the testes were abnormal and the hypertrophy of the hind-gut primordium continued unchecked into the second instar. In the third group there was no inhibition of the hypertrophy of any of the organs (stomach, salivary gland, and hind-gut primordium).

In larvae of all three categories, the first pair of glands fail to hypertrophy at the end of the second instar, but degenerate instead. The primordia of the remaining glands do not increase beyond their size in earlier stages and also degenerate. Plate 1, fig. D represents a set of mid-third instar glands compared with control fig. C (note that the magnifications are 100 and 48 respectively). Fig. E shows the extreme dwarfness of the lymph glands; their histological structure looks strikingly pathological.

This degeneration of the lymph-gland system was followed by exaggerated hypertrophic processes in the mid-gut, hind-gut, and salivary glands, so that all these organs become involved in nearly every individual. Plate 1, fig. F shows the early development of the gastric hypertrophy, which later in the third instar appears as shown in fig. G, being cytolysed to a homogeneous undifferentiated substance which has undergone melanization in certain parts. This proliferated mass can without exaggeration be referred to as a tumour or neoplasm. In some lethal larvae the histolysed tumour tissue served as a culture medium for the enclosed yeast cells, which grew to huge colonies and sometimes penetrated the wall of the stomach and formed large colonies in the haemocoele.

The production of the stomach tumour was preceded by the proliferation of the mid-gut basement cells, and ceased completely when these become melanotic and degenerate.

The neoplasmic activity of the hind-gut primordium is also inhibited in later stages of development, when the cells become melanized (Plate 1, fig. H). This process leads to the appearance of visible melanized structures attached to the hind-gut, which is characteristic of late lethal individuals. The salivary gland neoplasm may also cytolyse after the imaginal primordium has become involved in a similar reaction and undergone melanization; if this occurs the larva exhibits immovable black bodies in the anterior of the salivary glands (Plate 2, fig. I).

In the testes, the lymph-gland cells (oikocytes) usually show signs of degeneration at the end of the first instar, even in those individuals in which the glands themselves are most acutely affected. These cells become necrotic, and during the second instar appear melanized. There is no further growth or differentiation of the main body of the testes.

The tumourous condition of the larvae as described above results in occlusion of the alimentary canal, loss of appetite, and consequent death due to starvation.

It has been shown previously (Shatoury & Waddington, 1957*a*) that at the beginning of the second instar the proventriculus is colonized by certain cells ('hexagons') originating probably from the lymph glands. The hypothesis that these glands really are the source from which the cells originate is supported by the fact that in l-nib the proventricular oikocytes degenerate at the end of the second instar at the same time as the main lymph glands also undergo necrosis. In normal development the upper ring of the radial cells of the proventriculus becomes differentiated from the remainder by acquiring a strong basophilia. This takes place in the immediate neighbourhood of the hexagons. In lethal-nib the process comes to a halt immediately the hexagons degenerate (Plate 2, fig. J).

The degeneration of the lymph glands at the end of the second instar in these larvae is also followed by regressive changes in the ring gland. All the components of this organ appear cytolysed and structureless by the mid-third instar stage.

It has been pointed out that in the early generations of this stock, examined in 1953, the imaginal buds were almost completely absent. It is noteworthy that in larvae in which the cephalic imaginal buds have not been developed the optic glomeruli fail to appear. By the middle of the third instar the brain hemispheres have still only attained the size characteristic of the second instar, and their internal structures are very distorted and undifferentiated. At the beginning of the third instar the giant ganglion cells of the brain in these larvae become vacuolated and show signs of disintegration. It is hoped to collect further material which will allow a more precise description of the effect on the brain of the failure of the imaginal optic buds.

In these larvae from the earlier collections, in which the imaginal buds were absent, there was no development of a visible melanotic tumour attached to the hind-gut. However, sections of late larvae show evidences of tendencies towards tumourous overgrowths of the same general type as those described above. It seems that in the larvae of this strain the tendencies which act to restrain overgrowth, which were discussed by Shatoury (1955) in connexion with lethal malignant, were more powerful than they became in the later developments of the stock.

The l-nib/Muller-5 stock as it exists in Edinburgh was re-investigated in the summer of 1956. By this time the lethal phenotype had again suffered some alteration. Black tumours appeared in the hind-gut of many of the lethal larvae, which also showed swollen and occluded mid-guts. However, the degeneration of the lymph glands at the end of the second instar, although very noticeable, was not so complete as in the stock described above. Further, there was a typical development of the imaginal buds, although these were smaller than usual and by the middle of the third instar or at the time of death showed a large number of necrotic and pycnotic cells. In these larvae lymph-gland hexagons had moved into the optic buds, and by the mid-third instar exhibited the same peculiar features of cell degeneration as are found simultaneously in the lymph glands themselves. There was not the usual clear deposition of melanin in the cells, but instead they become pycnotic and disintegrate to form rather featureless masses which stain deeply in haematoxylin.

The development of gastric tumours in lethal malignant

Shatoury (1955) has described the syndrome of effects produced by another sex-linked lethal, lethal-malignant. In this stock the lymph glands, at the end of the third instar, hypertrophy and undergo very extensive cell proliferation. The cells released from them infiltrate into the imaginal buds, metastasise along the ventral nerve-cord, and attack the fat bodies, exhibiting the behaviour of a true malignant tumour. If, as seems to be suggested by the phenomena in l-nib, the hypertrophy of the gastric epithelium is only held in check by the presence of healthy lymph glands, it seems possible that the abnormal condition of these organs in lethal malignant might be followed by some pathological situation in the stomach. Little attention had been paid to this organ in the earlier investigations of this stock and a new investigation of the point was therefore made. A series of larvae were fixed at 10-hour intervals, starting from the 5-day-old stage.

The structure of the stomach wall appeared to be quite normal until the time, late in the third instar, at which the lymph glands begin their abnormal hypertrophy and proliferation. At this stage the cells lying immediately against the basement membrane (the basement cells, see Shatoury & Waddington, 1957*b*) undergo considerable multiplication (Plate 2, fig. K). At the same time there is a swelling of the inner ends of the large epithelial cells which line the gut lumen (fig. L). These swollen ends become strongly basophilic and eventually break free into the gut-cavity. The growth of these cells eventually takes the form of true cell proliferation, the gut becoming full of cytoplasmic masses, most of which contain nuclei (fig. M).

In the later stages of the development of lethal malignant larvae, the cells of the lymph glands and the primary tumour cells released from them are 'antagonized' and suffer degenerative changes, one striking symptom of which is the deposition of melanin. At this time similar changes may occur in some of the proliferated basement cells of the stomach wall (fig. N). The melanin formed in these cells is of a reddish tint in sections and is easily masked in preparations which have been counterstained with eosin. When these degenerative changes occur in the basement cells, the proliferation of the main epithelium of the midgut ceases and the free cells or cytoplasmic masses in the gut lumen break down to form a homogeneous undifferentiated mass, in which in a very few cases the deposition of melanin takes place.

DISCUSSION

The conditions found in these two lethal mutants provide some confirmation for two suggestions which arose from the examination of the course of development during the larval period of wild type *Drosophila*. The first of these concerns the inductive action of the oikocytes given out from the lymph glands. Shatoury & Waddington (1957*a*) have described how these cells arrive at the testis and proventriculus, among other organs, and argued that appearances suggested the hypothesis that the oikocytes stimulate the further differentiation of the tissues with which they come into contact; in the testis, the formation of primary spermatocytes, and in the proventriculus of the 'upper ring' of the imaginal primordium. It is quite in line with this that we should find that when, in lethal-nib, the testicular oikocytes degenerate towards the end of the first instar, no further development of the testis occurs, while the necrosis of the proventricular hexagons in the third instar is followed by a failure of the differentiation of the upper ring.

In normal development it is also found that the gastric epithelium, and the imaginal primordia of the hind-gut and salivary glands, undergo processes of growth and regression in phase with the hypertrophy, degeneration, and regeneration of the lymph glands. The fact that in lethal-nib the early degeneration of the lymph glands is followed by extreme hypertrophy, amounting to neoplastic proliferation, of the tissues of the intestinal tract, strongly suggests that the growth of these tissues is normally under the control of the lymph glands. A similar conclusion is suggested by the phenomena seen in lethal-malignant, in which a different type of lymph gland degeneration, occurring at the end of the third instar instead of at the end of the second, is again followed by neoplastic proliferation of the gastric epithelium.

The most convincing proof of the suggested relation between the lymph glands and the intestinal growths would be the demonstration that normal lymph glands, injected into lethal-nib larvae, could inhibit the hypertrophy of the gastric and other tissues. Some preliminary experiments of this kind have already been performed by one of us (H. H. El S.). The results will be described in detail in a later communication; here it can be stated that the data so far to hand agree entirely with expectation, and that it indeed seems possible to suppress completely the appearance of melanotic growths in lethal-nib larvae by the injection of normal lymph glands into the early third instar stage.

It is noteworthy that in the lethal-nib stock, as it existed at the time of the main study in 1955, there was considerable variation in the degree to which the lymph glands were affected, and that there was a regular parallelism between the degeneration of these glands and the hypertrophy of the other organs. Thus even in individuals in which the glands were relatively normal till late in the second instar, the oikocyte component of the testis was always degenerate from an early stage. The next most easily affected organ was the hind-gut primordium, while the mid-gut and salivary glands only exhibited hypertrophy when the lymph glands were more severely damaged. On the hypothesis that the glands give out a hormone which controls the growth of these organs, one would have to suppose that the hormone level required for normal development of the testis cells is higher than that for the hind-gut, which itself requires a higher level than the mid-gut.

The relations between the lymph glands, the oikocytes in various organs, and the epithelia of the intestinal canal are clearly very complex, and much more remains to be learnt about them. For instance, it will have been noticed that the two rather different types of lymph gland degeneration which occur in the two lethals are followed by different kinds of intestinal tumour. In lethal-malignant the proliferation of the basement cells seems to be a more marked feature of the syndrome than it is in lethal-nib. Shatoury (1955) has suggested that the effect of the lymph glands on the gastric epithelium is always a secondary one, the primary effect being on the basement cells, while these in their turn provoke the hypertrophy of the main gastric epithelium. This hypothesis is probably the simplest which can be made, and there is nothing in the present observations which contradicts it, since even in lethal-nib there is some proliferation of these cells as well as of the main epithelium.

It seems most probable that the ring gland is also involved in some way in this system of interactions. It is noteworthy that it degenerates in lethal-nib. But we still know very little of its relations with the lymph glands.

The manner of death of lethal-nib shows many resemblances to that of lethal-meander, a mutant which has been described in particular by Schmid (1949) and Hadorn (1955). This lethal dies during the third instar, apparently from an inability to absorb nourishment. Schmid showed that a very closely similar phenocopy can be produced by starvation of wild-type larvae. Hadorn (1955) describes a detailed study, by himself and Chen, of the free amino acids in the mutant and the phenocopy, finding a broad parallelism but also some differences between the two in their divergences from wild type. Unfortunately no information has been published on the internal anatomy of this mutant. If the inability of the mutant to feed were to be due to occlusion of its gut by excessive hypertrophy of the gastric epithelium, it is clear that we might expect the free amino-acid pool to be abnormal not only on account of the starvation but also because of the proliferation of an abnormal tissue. It would seem important that the refined biochemical methods of investigation should be supplemented by the more old-fashioned but still basic histological techniques.

It appears difficult, in the present state of our knowledge, to form any definite opinion about the relations between these *Drosophila* tumours and the neoplasms of vertebrates. We have seen that the normal development of wild-type individuals involves periodic phases of hypertrophy in several tissues, such as the gastric epithelium and the hind-gut and salivary gland primordia; and opposing the tendencies towards proliferation or hypertrophy there are antagonistic tendencies which bring about periodic phases of regression. Shatoury (1955) has already drawn attention to the existence of these two opposing tendencies in connexion with the hypertrophy and regression of the lymph glands themselves; when, in this organ, the balance becomes upset in lethal-malignant, the result is the exaggerated proliferation of lymph-gland cells which form an apparently malignant tumour. The cells of such primary lymph-gland tumours are normally small and fairly rapidly dividing elements, and can perhaps be compared without too much difficulty to the tissue cells of a vertebrate. The hypertrophic tissues with which we have been concerned in this paper are of rather a different type. They are derived from tissues, such as the gastric epithelium, which are polytene in their nuclear constitution. Their hypertrophy in the first instance leads to the setting free of non-nucleated lumps of cytoplasm; only in the exaggerated conditions seen in the lethal stocks do nuclear masses frequently appear in these cytoplasmic blebs. It is much less easy to see how such phenomena are related to vertebrate cancers, though one would certainly feel that the two types of phenomena belong to the same general area of cell biology.

In the meantime, we seem to be gradually discovering the outlines of a rather novel type of epigenetic organization. It should perhaps be expected that a holometabolous insect, which develops into a larva which carries concealed within it the rudiments of an imaginal body totally dissimilar to the larva itself should utilize to this end a system of developmental interactions unlike anything known in animals whose development is more straightforward. It certainly seems difficult to think of close parallels for the 'oikocytes' which, leaving one and the same gland, move to different larval organs and appear to activate a number of different types of differentiation; while the balance between tendencies to hypertrophy and regression, one or other of which may temporarily get the upper hand, also seems to give a characteristic flavour to *Drosophila* development. It will be interesting to discover how far similar processes occur in other *Diptera*.

SUMMARY

1. A sex-linked factor in *Drosophila*, lethal-no-imaginal-buds (l-nib) produces two main effects: reduction, or even complete absence of the imaginal buds; and excessive proliferation of the epithelium of the stomach, leading to occlusion of the gut and death from starvation. The intensity with which these two effects are expressed varies independently in different stocks. This paper is primarily concerned with the formation of the gastric tumour.

2. The first pair of lymph glands, after their breakdown in the later part of the first instar, usually regenerate only incompletely. With increasing abnormality of the glands, there was an increasing tendency for excessive proliferation, or for cellular degeneration, in the testes, hind-gut primordia, and stomach, in that order.

3. In severely affected larvae, the lymph glands degenerate at the end of the second instar. This is followed by, and is thought to be the cause of, a strong hypertrophy of the gastric epithelium, which leads to the filling up of the gut lumen with cells and anuclear cytoplasmic masses. It is probable that a preliminary stage in the development of this hypertrophy is the proliferation of the cells of the mid-gut imaginal primordia.

4. Similar proliferation and/or hypertrophy occurs in the hind-gut and salivary gland primordia.

5. The cells of all these proliferations eventually undergo degenerative processes involving the deposition of melanin, when they become converted into melanotic 'tumours'. These are usually anchored to their places of origin, in contrast to the free tumours found in some other *Drosophila* stocks.

6. Preliminary experiments indicate that the development of the proliferations can be suppressed by the injection of normal lymph glands into early third instar larvae.

7. In individuals from which the cephalic buds are missing, there is no development of the optic glomeruli in the brain.

8. In the stock lethal-malignant (Shatoury, 1955) the lymph glands undergo pathological changes of a different type; they become excessively hypertrophied in the late third instar, and develop into a metastasizing tumour which eventually undergoes various degenerative changes. In this stock also the gastric epithelium begins to exhibit excessive hypertrophy at the same time as the lymph

glands become abnormal. A full gastric tumour may develop; in this case the basement cells are involved in addition to the main epithelium.

9. It is suggested that the complex interrelations between the lymph glands, the migratory cells given off from them (oikocytes), and the apparently hormonally controlled proliferative tendencies of tissues such as the gastric epithelium, constitute a novel type of epigenetic system, which may be special to the larvae of holometabolous insects or even of *Drosophila* alone.

ACKNOWLEDGEMENTS

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EXPLANATION OF PLATES

Abbreviations: CSAN, stroma from cytolysed proliferations of salivary primordium; CT, cytolysing cells of tumour; DFO, degenerated foregut oikocytes; GE, GP, gastric epithelium; GTC, gastric tumour cells; HEP, hypertrophied epithelium; HGP, hind-gut primordium; HMO, hypertrophied basement cells; HT, heart; LFP, lower ring of fore-gut primordium; LG, degenerating main body of lymph gland; MGP, mid-gut imaginal primordium; MLG, melanized lymph-gland cells; MMO, melanized basement cells; MO, basement cells; MSHG, melanized cells of hind-gut primordium; MSSA, melanized salivary imaginal primordium; MT, melanized cells of tumour; OES, oesophagus; PC, pericardial cell; PV, proventriculus; SA, main epithelium of gland; TC, terminal cytoplasmic mass; TR, trachea; UFP, upper ring of fore-gut primordium; WC, wreath cell of Weismann's cell chaplet.

PLATE 1

FIG. A. Wild type, and FIG. B, lethal-nib late third instar larvae at same magnification.

FIG. C. Heart and lymph glands of mid-third instar wild type larva. ×48.

FIG. D. Same of l-nib larva, ×100. *Note relative magnifications of Figs. C and D.*

FIG. E. First lymph gland of third instar l-nib larva. ×470.

FIG. F. Late second instar l-nib, showing beginning of hypertrophy of gastric epithelium. ×90.

FIG. G. Occluded mid-gut of third instar l-nib larva. ×170.

FIG. H. Melanized tumour of hind-gut primordium of l-nib. ×100.

PLATE 2

FIG I. Anterior end of salivary gland of third instar l-nib, showing melanization of imaginal primordium. ×100.

Fig. J. Proventriculus, late third instar of l-nib, showing degeneration of fore-gut oikocytes and failure of differentiation of upper ring.

Fig. K. Hypertrophy and proliferation of basement cells in third instar lethal-malignant. × 370.

Fig. L. Early stage in hypertrophy of gastric epithelium in third instar l-mal. × 190.

Fig. M. Occlusion of stomach in late third instar l-mal larva. × 90.

Fig. N. Wall of mid-gut, l-mal larva. × 370.

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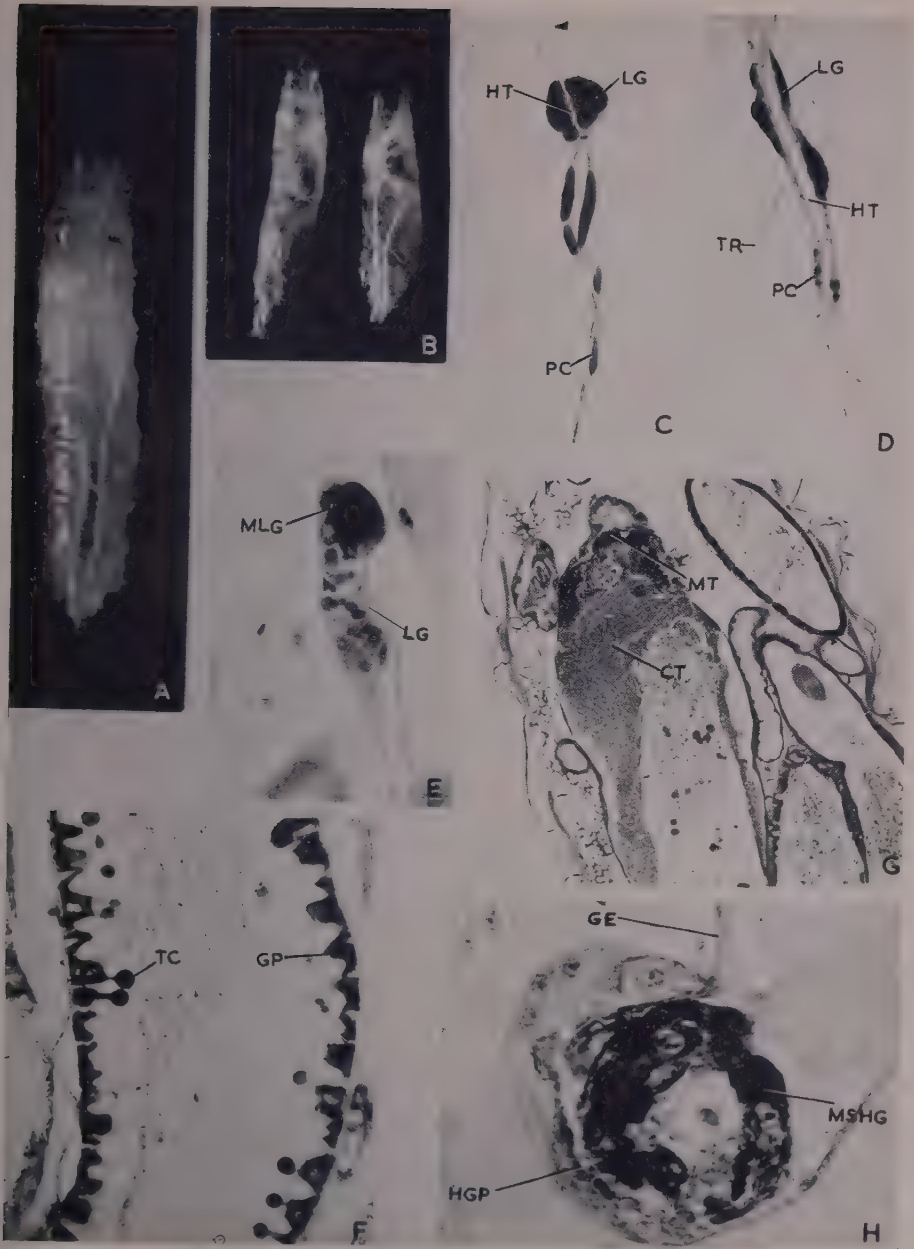

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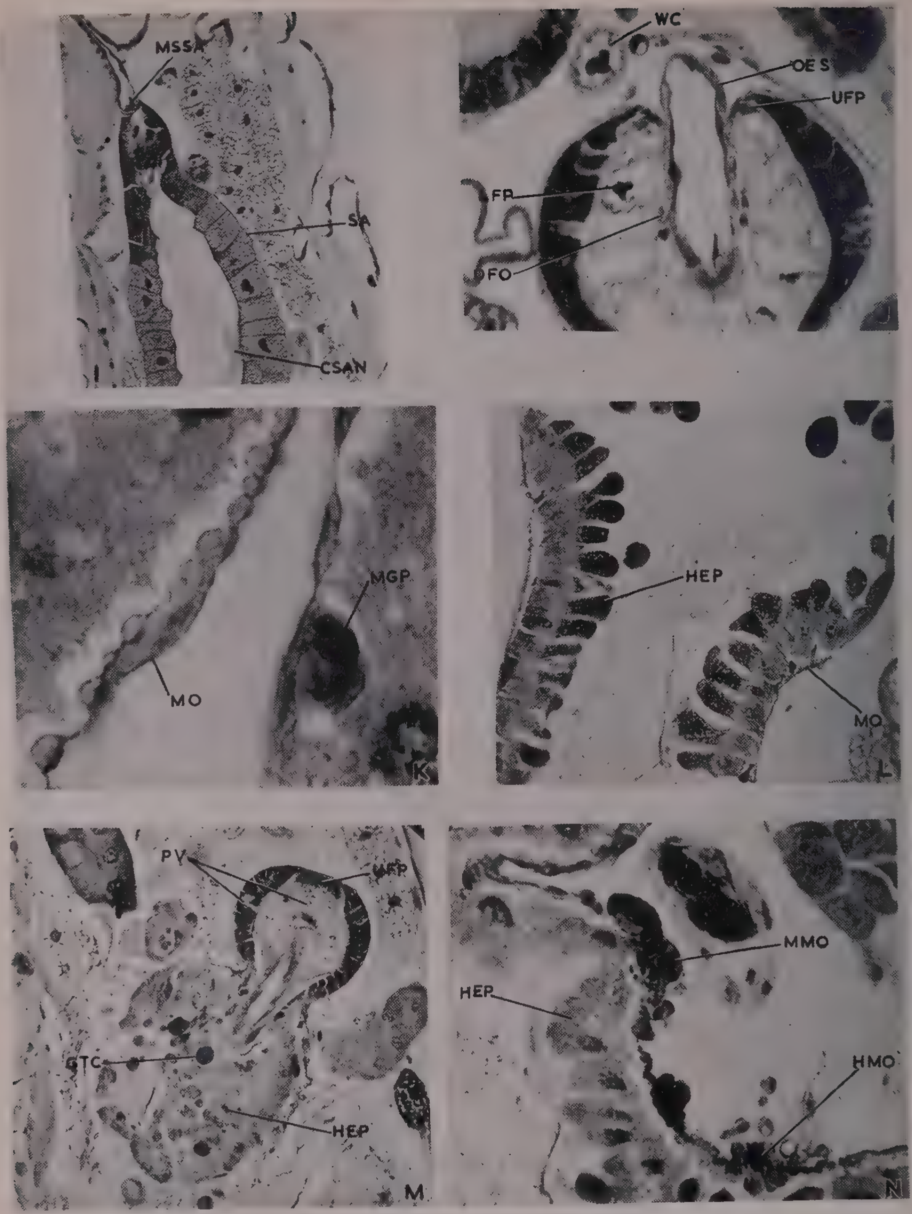
Plate 1



H. H. EL SHATOURY *and* C. H. WADDINGTON

Plate 2

The Penetration of Phosphate into Whole and Dissected Amphibian Embryos

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INTRODUCTION

THIS work on the introduction of radioactive phosphate into amphibian embryos was carried out as a necessary preliminary to research on embryonic phosphate metabolism, in which 32P was to be used as tracer. It has an interest of its own for two reasons.

In the first place, recent work on the penetration of phosphate into cells in general suggests that this phenomenon is not simply a result of diffusion, but is related to the metabolism of the cell (cf. Danielli, 1954), and may be mediated by a 'carrier' at the cell membrane (cf. Sutcliffe, 1954, and Russell, 1954, with reference to plant cells, and Sacks, 1948, and Popjak, 1950, for mammalian cells). If these hypotheses are confirmed, differences in phosphate uptake which may appear among embryonic cells in the course of development may be regarded as indicative of metabolic differences, and even possibly of differentiation of membrane structure. The importance which such a membrane differentiation might have in development has been presented in abstract terms by Weiss (1953). Therefore, in spite of the fact that amphibian embryos do not, in general, need phosphate from an outside source for their development, if different stages and different types of cells show differential permeability to the ion, this may indicate interesting metabolic differences.

In the second place, results for Amphibia may be compared with the extensive data available on the subject of phosphate uptake by echinoderm embryos. Marine embryos such as echinoderms normally absorb considerable quantities of phosphate from sea-water during their development. This phosphate absorption, which is a necessary function of the embryo, is very sensitive to such factors as change of temperature and the presence of metabolic inhibitors (cf. Discussion). It is interesting to contrast the behaviour of these embryos with that of amphibian embryos, which have evolved a relative independence of their environment.

So far as Amphibia are concerned, little work has been published. Villee &

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Duryee (1951) kept unfertilized eggs of *Triturus pyrrhogaster* in a medium containing 1 μC. per ml. of 32P, and after 6–24 hours were able to fractionate the labelled compounds chemically. Hansborough & Denny (1950, 1951) found that phosphate penetrated embryos of *Rana pipiens*. Measurements of the penetration rates were not given in either of these papers. Kutsky (1950) reported only a negligible penetration of phosphate into *R. pipiens* embryos without giving details.

MATERIALS AND METHODS

Eggs. The axolotl eggs used in the earlier experiments were obtained in spring by bringing together male and female animals previously kept apart. The eggs of *Xenopus laevis* were all obtained by injection of the parents with 'Pregnyl' (chorionic gonadotropin).

Isotope. The 32P was supplied as sterile, carrier-free, radioactive orthophosphate in a small volume of dilute hydrochloric acid.

Media. Radioactive culture media were made by adding the isotope to Holtfreter solution which had been boiled and cooled. This medium was then neutralized with sodium bicarbonate. If whole embryos were to be incubated, one-third-strength Holtfreter solution was used; if dissected parts of embryos, full-strength solution. When dinitrophenol was used, it was added before boiling the solution.

Concentrations of from 5 to 10 μC. 32P per ml. of solution were used in these experiments. In each case samples of the medium were evaporated on planchettes and the content of 32P was estimated with an end-window Geiger–Muller counter.

The 'carrier-free' 32P contains a very small quantity of 31P, which varies from one batch to another. The total 31P + 32P is too small to estimate chemically. The specific activity (i.e. counts per minute of 32P per mg. phosphate) of the media could therefore only be standardized by dilution with a known, larger quantity of 31P. The number of counts per minute per unit weight of phosphate could then be stated, and the actual amount of phosphate taken up by the embryos could be calculated. However, as the embryos take up so little phosphate, it seemed advisable to add the isotope mixture to a perfectly phosphate-free solution, sacrificing the possibility of knowing the exact amount of phosphate which had entered the cells to the retention of the very high specific activity of the initial 'carrier-free' solution.

The concentrations and uptake of phosphate are therefore expressed in counts per minute per millilitre of solution or per embryo.

Experiments. Before the embryos were incubated in the radioactive medium, the jelly was removed with forceps, and they were washed by transference through four dishes of sterile one-third-strength Holtfreter solution.

Dissections of embryos were performed with the point of the forceps in full-strength Holtfreter solution on a layer of agar. When the whole group had been cut up as quickly as possible 10 minutes were allowed to elapse before the fragments were transferred either to the 32P medium or to a larger agar-lined dish to await later incubation. The first phase of closure of the wound was therefore completed before the fragments were moved.

The temperature was kept constant during the whole of any one experiment, but was not the same for all experiments.

At the end of the incubation in the 32P medium the embryos or fragments were pipetted into a large volume of sterile Holtfreter solution, and washed by repeated transferences until the washing medium was no longer radioactive. This usually took from 10 to 15 minutes.

Fragments of embryos were then pipetted individually on to planchettes and dried for estimation of 32P by means of an end-window Geiger–Muller counter.

Early experiments with axolotl embryos showed that their vitelline membranes do not become radioactive, so that there was no need to remove these before drying the embryos. The vitelline membranes of *Xenopus* embryos usually have adhering strands of jelly, and become extremely radioactive in a medium containing 32P. Before the whole *Xenopus* embryos were dried, therefore, the membrane was removed. This operation was usually delayed for a few hours for the sake of convenience. It was found that there was no measurable loss of the 32P once it had been taken up.

The uptake of 32P was so small that it would have been almost impossible to estimate the 32P content of each embryo individually. Groups of from two to six were therefore dried together on each planchette. These formed so very fine a film of material that it was not necessary to correct the results for self-absorption of electrons.

Two counts were taken of each sample, each of these being high enough by itself to keep the counting error below 3 per cent. The counter was checked daily with a uranium sample. All counts were corrected for radioactive decay.

Cytological technique. The following was found most satisfactory for dissected parts and for whole embryos.

Fixation overnight in 20 per cent. trichloracetic acid at about 2° C. (in the refrigerator). *Dehydration* in 70 per cent. ethanol at 2° C. after a rinse in distilled water at the same temperature; then allowed to rise to room temperature, transferred after 1 hour to 90 per cent. ethanol and after a further hour to 100 per cent. ethanol. After a total of 2–3 hours in 100 per cent. ethanol (two changes) *cleared* in chloroform for 2–3 hours (or overnight). Embedding in wax in the usual way, with not more than two changes each of three-quarters of an hour in hot wax. Some whole embryos, including samples of those treated with dinitrophenol, were embedded and sectioned in the same way.

Controls. The possibility of radiation damage to the embryos was carefully checked. Embryos which had been incubated in the radioactive medium were compared with two kinds of controls, A and B.

A. Random groups of the same batch of eggs were kept in open dishes in tapwater. Among these embryos many hatched, some were reared to metamorphosis, and occasionally a few were reared to maturity. Some were infertile or had suffered damage by the parent toads, and in some spontaneous anomalies such as microcephaly and exogastrulation occurred. Mortality was fairly high.

B. A group of embryos was dejellied, washed, and cultured in sterile conditions in one-third-strength Holtfreter solution. As in the experimental group, all defective or abnormal embryos were carefully excluded at the time of selection.

The frequency of abnormality and mortality was the same in the control group B as in the experimental groups (usually about 1 to 2 per cent.) and was much lower than in control group A.

Embryos which had been kept in a radioactive medium for a long time did not show a greater liability to defect than those incubated for a shorter time. The rate of development of irradiated embryos was the same as that of the controls. No signs of radiation damage were seen in sections of embryos and fragments incubated in 32P.

RESULTS

The uptake of phosphate by whole embryos at different stages of development. In preliminary experiments embryos of *Siredon mexicanum* were incubated continuously in radioactive medium from the uncleaved egg stage until the tail-bud stage, and a number of embryos was taken out at each stage for estimation of the 32P taken up. This technique gave substantially the same results as subsequent experiments with short periods of incubation, but was unsatisfactory in the first place because of the prolonged irradiation, and in the second because bacteria eventually invaded the medium. Towards the end of the experiments the embryos showed signs of cytolysis, and by shedding cells actually lost radioactivity. Several unfertilized eggs which were included in these experiments showed no signs of cytolysis throughout the long period in radioactive medium. In contrast to the developing embryos they were almost completely impermeable to phosphate. For instance, in one experiment, after 46 h. 20 min. in the radioactive medium, three mid-gastrulae had taken up 423, 448, and 509 counts per minute of 32P respectively, three unfertilized eggs only 15, 10, and 20 counts per minute respectively.

Two experiments with 1-hour incubations in radioactive medium were carried out. There were inexplicable differences between the two sets of results, but in both it appeared that there was a rather higher uptake during the cell movements of gastrulation and neurulation, followed by a definite fall in penetration rate after closure of the neural folds. An experiment with a single batch of embryos of *Triturus alpestris* gave similar results.

These first experiments were carried out in the laboratory of Professor J. Brachet of Brussels University. Subsequent experiments were carried out in London with a different stock of *Siredon mexicanum*. The embryos produced by this stock proved to be totally impermeable to radioactive phosphate.

Embryos of *Xenopus laevis* were therefore used for further experiments.

Results are given in Table 1. These embryos develop so quickly that the 2-hour incubations cover almost the whole period of development from cleavage to formation of the tail-bud. For instance, the mid-gastrula (yolk-plug) stage has reached the early neurula stage at the end of the incubation period. The numbers in each group as given in the table represent numbers of radioactive samples counted; each sample consisted of several embryos.

TABLE 1

The uptake of phosphate by whole embryos of Xenopus laevis *expressed in mean counts per minute of 32P per embryo taken up in 2 hours with standard error*

The numbers in brackets represent the numbers of radioactive samples, each consisting of several embryos. Stage numbers after Nieuwkoop & Faber (1956). A, B, and C represent subgroups of the same batch of eggs.

	Stage of embryo during incubation in 32P					
Experiment	*Cleavage St. 6–9*	*Blastula St. 9–10*	*Early gastrula St. 10–11*	*Mid to late gastrula St. 11–13*	*Neurula St. 13–17*	*Tail-bud St. 22–23*
1	1·9±0·3 (9)	2·4±1·4 (6)	8·4±1·4 (3)	4·3±0·9 (3)	2·2±0·4 (3)	0·45±0·05 (2)
2	3·0±0·4 (4)	0·9±0·1 (4)	1·5±0·2 (5)	1·2±0·1 (4)	1·0±0·1 (5)	0·9±0·3 (4)
3A	5·6±0·8 (4)	6·4±0·4 (6)	10·5±1·4 (6)	12·6±1·2 (5)	17·6±2·6 (5)	5·2±1·1 (6)
3B	6·8±1·1 (5)	7·6±2·2 (6)	12·8±2·1 (6)	14·9±2·4 (6)	10·1±1·5 (5)	2·0±0·4 (6)
3C	12·1±1·8 (5)	—	11·1±1·5 (7)	15·8±1·1 (6)	6·7±0·7 (7)	2·5±0·2 (6)
4A	6·1±0·6 (5)	15·2±2·3 (7)	18·1±4·2 (7)	18·9±3·9 (6)	16·6±1·7 (7)	4·4±0·4 (6)
4B	11·1±1·8 (6)	21·0±1·5 (7)	18·2±2·1 (7)	10·2±1·4 (5)	16·6±1·5 (5)	2·6±0·3 (6)
4C *	13·5±1·0 (5)	4·6±0·9 (7)	19·8±3·6 (7)	4·4±0·4 (6)	25·4±0·1 (7)	4·6±0·8 (6)
5A	4·4±0·5 (7)	2·8±0·6 (7)	1·8±0·3 (7)	4·0±0·5 (8)	12·0±2·4 (8)	2·2±0·3 (8)
5B	6·5±0·7 (8)	1·9±0·2 (7)	6·5±0·6 (8)	5·4±0·6 (8)	14·8±3·2 (9)	2·2±0·3 (8)
5C	4·7±0·4 (8)	1·8±0·2 (10)	11·2±1·0 (8)	13·6±2·0 (7)	15·6±1·6 (8)	—

* A fresh medium used for each stage.

Since a different sample of 32P was used for each experiment and each sample has a different specific activity, the absolute values are not comparable from one experiment to the next. The variation between different experiments is high, which is understandable in view of the minute quantities of material involved, but the relative values for different stages show the same sort of pattern in the various experiments. The gastrula and neurula stages in general have the highest uptake, the tail-bud stage always takes up least phosphate.

The penetration of phosphate into unfertilized and uncleaved but fertile eggs could not be measured, since it is almost impossible to remove the jelly from these without causing severe injury.

In most experiments one dish of medium was used for all the stages. The uptake was so slight compared with the concentration in the medium that depletion of the 32P was negligible, but it seemed possible that the results might be falsified by growth of bacteria. In two experiments, therefore, a fresh dish of medium was used for each stage for one of three groups (see Table 1). There was no obvious difference in the group so treated. The estimated radioactivity of the various media was as follows: experiment 1: 554,000 counts per min. per ml.; experiment 2: 1,338,000 c./m.; experiment 3: 1,474,000 c./m.; experiment 4: 1,584,000 c./m.; experiment 5: 1,390,000 c./m. (approx. 10 μC. per ml. in the last three).

Abnormal embryos. Two series of *Xenopus* embryos were incubated in the radioactive medium throughout development, and samples were taken at intervals as in the preliminary axolotl experiments. There was no cytolysis or appearance of infection in these, but the results were unsatisfactory in that variability in uptake became progressively greater at late stages. The interest of these experiments is that among the embryos, all of which had been apparently normal when put into the medium at the blastula stage, were three which developed into microcephalic monsters with a single median eye. The phosphate uptake of these was measured separately and found to exceed to a great extent the uptake of normal embryos (Table 2).

TABLE 2

The uptake of phosphate (counts per minute, c./m.) by microcephalic embryos of Xenopus laevis *compared with that of normal embryos. Mean counts per minute of 32P with standard error; stages after Nieuwkoop & Faber (1956)*

Stage taken out of 32P	*Time in 32P*	*c./m. 32P taken up by microcephalic*	*c./m. 32P in normal embryo*	*No. of normals*
	(hours)			
14	14	346	45±18	7
16	16	357	49±20	8
18	18	947	53±18	10

Concentration of phosphate, pH, and other conditions. In general the pH of the radioactive medium was adjusted to 7, but in one experiment groups at pH 6·0 and 7·2 were compared. There was no difference in uptake except that the group at pH 6·0 gave more variable results. In the same experiment, media prepared from the same sample of isotope diluted to different concentrations were prepared. Over a range of 3–10 μC. per ml., the ratio of 32P uptake to concentration of 32P in the medium was roughly constant for a given stage, indicating an uptake in direct proportion to concentration of the medium (Table 3). Each value was derived from about ten embryos. A second smaller experiment gave similar results. In the preliminary work with axolotl embryos such proportionality was also found.

TABLE 3

The ratio of uptake of 32P to the concentration of the medium Xenopus *embryos*

	Ratio of uptake to medium concentration					
	Media at pH 7·2			*Media at pH 6·0*		
μC. 32P per ml. added to medium	*5 μC.*	*7 μC.*	*10 μC.*	*5 μC.*	*7 μC.*	*10 μC.*
Blastula	1·8	2·0	1·7	1·8	2·3	1·8
Gastrula	4·4	4·5	5·2	4·0	2·0	3·7
Yolk-plug	5·3	8·5	7·9	5·4	3·4	5·9
Neurula	5·1	4·2	4·7	14·0	5·2	1·6
SUM OF ALL STAGES .	17	19	19	25	13	13

Factors such as the degree of crowding, depth, and volume of the medium, which might affect metabolism and therefore also phosphate penetration, were kept constant for any one experiment, and varied little from one experiment to another. A few experiments in which these conditions were varied showed that phosphate uptake was in fact affected. More crowded embryos, for instance, took up less phosphate.

The effect of injury. When fragments of gastrulae and neurulae were incubated in radioactive media, whole embryos of the same stage were relieved of their vitelline membranes, being punctured in the process, and incubated with the halves for comparison (Tables 6 and 7). In the period from immediately after cutting until 2 hours later, the amount of phosphate taken up was similar to that taken up by the fragments, and was much higher than that which penetrates intact embryos with vitelline membranes. Subsequently the rate of phosphate uptake diminished, though not to such a low level as that of a normal embryo at this stage.

In a few experiments normal embryos in their membranes and injured embryos without membranes were incubated in the same medium and so directly compared. For instance, in one experiment, the uptake of normal embryos, incubated for 2 hours in the medium, was 0·9 ± 0·2 counts per minute 32P, while that of the injured embryos was 15·2 ± 1·3 counts per minute (15 embryos in each group, 5 radioactive samples).

It seems unlikely that the vitelline membrane actually hinders entry of phosphate. In the case of axolotl embryos, which can be removed more easily from the membranes, without injury, it was found that taking off the membranes had no effect on phosphate uptake. It cannot be assumed either that the radioactive medium simply flows into the wound, since the first rapid phase of closure of the wound was completed before the embryo was transferred to the medium, and in the experiments with fragments, healing appeared perfect before the third incubation period (i.e. from 4 to 6 hours after cutting) usually even before the beginning of the second incubation (from 2 to 6 hours after cutting). The increased uptake of phosphate appears to be connected either with the exposure at the surface of cells usually covered, or else with the metabolic changes involved in recovery from the wound.

TABLE 4

Dry weights of half-embryos of Xenopus laevis *as per cent. of whole embryos*

Stage	*Dorsal half*	*Ventral half*
	(per cent.)	(per cent.)
Blastula	39·5	60·5
Gastrula	48·5	51·5
Neurula	32·0	68·0

TABLE 5

The uptake of phosphate by Xenopus *half-blastulae expressed in mean counts per minute 32P taken up in the time stated with standard error*

In Experiment I each group was divided into subgroups 1 and 2, which were incubated separately.

Experiment I (February 1954). Medium 794,000 c./m./ml. 32P at 17° C.

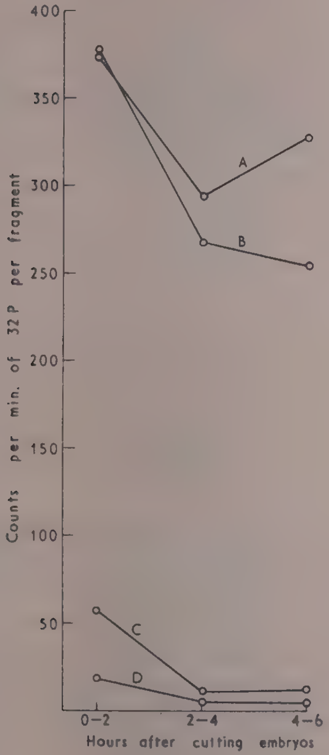
Time after cutting (hours)	*Animal half (D)*	*No. of embryos*	*Vegetative half (V)*	*No. of embryos*	*Ratio D/V*
0–2 1	40±6	23	57±8	21	0·70
2	19±4	12	58±15	9	0·33
2–4 1	34±12	22	20±6	17	1·70
2	5±1	15	11±2	11	0·45
4–6 1	9·5±4	18	9±2	18	1·06
2	5±1	23	12±2	20	0·42

Experiment II (March 1954). Medium 324,000 c./m./ml. 32P at 19° C.

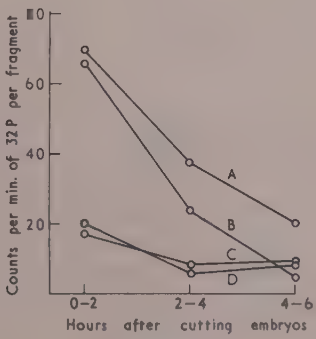
Time after cutting (hours)	*Animal half (D)*	*No. of embryos*	*Vegetative half (V)*	*No. of embryos*	*Ratio D/V*
0–2	20±2	20	17±1	14	1·18
2–4	5·5±1	23	8±1	20	0·69
4–6	8±2	21	9±2	14	0·89

The uptake of phosphate by fragments of Xenopus *embryos.* The proportion of the original embryo present in each fragment was estimated as follows. Batches of ten fragments or whole embryos were weighed after being dried at 115° C., and the mean dry weight of one embryo or fragment was calculated. The average values for two batches of eggs are given in Table 4.

The blastulae were cut round the equator, half-way between animal and vegetative poles; the difference in weight is presumably due to the presence of the blastocoel in the animal half. The gastrulae were cut through the middle of the blastopore at the horse-shoe stage, so that the future dorsal halves were separated from the future ventral halves. The neurulae were cut so as to include in the dorsal half all the neural plate except for the tail region, which is curled round ventrally at this stage. Almost all the endoderm was included in the heavier ventral half.



TEXT-FIG. 1



TEXT-FIG. 2

TEXT-FIG. 1. Comparison of the uptake of phosphate by fragments of *Xenopus* gastrulae and blastulae incubated in the same radioactive medium. A, ventral half of gastrula; B, dorsal half of gastrula; C, vegetative half of blastula; D, animal half of blastula

TEXT-FIG. 2. Comparison of the uptake of phosphate by fragments of *Xenopus* neurulae and blastulae incubated in the same radioactive medium. A, dorsal half of neurula; B, ventral half of neurula; C, vegetative half of blastula; D, animal half of blastula

The radioactive medium used for the first experiment, with half blastulae (Table 5) was made up from the same sample of 32P, diluted to the same extent, as that used for the experiment with half-gastrulae, the results of which are shown in Table 6. For this reason these values for the properties of blastulae and gastrulae are directly comparable. The results for the second experiment in Table 5 (half-blastulae) are for the same reason directly comparable with those of experiment II in Table 7 (half-neurulae). It can be seen that the slight tendency of whole gastrulae and neurulae to take up more phosphate from the medium than blastulae is greatly accentuated when fragments of these stages are used. The half-blastulae take up less phosphate than half-neurulae, and much less than half-gastrulae. These comparative data are shown in Text-figs. 1 and 2.

Table 6

The uptake of phosphate by Xenopus *half-gastrulae, expressed in mean counts per minute 32P taken up in the time stated with standard error*

Each group was divided into subgroups 1 and 2, which were incubated separately. Medium 883,000 c./m./ml. 32P at 18° C.

Time after cutting (hours)		*Dorsal half*	*No. of embryos*	*Ventral half*	*No. of embryos*	*Injured whole embryos*	*No. of embryos*	*Ratio D/V*
0–2	1	313±41	12	340±45	11	390±76	5	0·92
	2	378±38	16	374±32	17	331±51	7	1·01
2–4	1	198±13	19	223±20	17	211±47	7	0·89
	2	268±23	18	294±18	17	289±46	7	0·91
4–6	1	181±17	15	253±27	13	82±47	6	0·72
	2	254±26	23	328±40	13	100±46	5	0·77

Two further experiments with half-neurulae fully confirmed the results given in Table 7. One further experiment each with blastula and gastrula fragments confirmed the results of Tables 5 and 6, except for one difference in the blastula results. This experiment was carried out at a higher temperature, so that development was more rapid, and during the period from 4 to 6 hours after cutting the ventral halves formed deep blastopores. During this period the uptake of 32P was not only much higher than that of the animal halves (72 ± 11 c./m. as compared with 12 ± 4) but also much higher than the uptake during the 0–2 hour period (29 ± 5 c./m.).

Half-blastulae (Table 5). The halves collapsed after cutting, but within 5 minutes had rolled into balls, with a small group of yolky cells protruding at the cut surface. These cells fell away or were covered in within 2 hours, so that healing was complete before the beginning of the second period of incubation (i.e. from 2 to 4 hours after cutting). Sections showed that cell-division continued in the fragments, bringing about the normal diminution of nuclear size, from about 20 μ diameter at the time of cutting, to a value approaching 8 μ, which is the normal nuclear diameter in stages after the beginning of gastrulation, and in adult skin.

Fragments from experiments I and II (Table 5) were kept for several days after cutting. A small abortive blastopore formed in vegetative halves, while epibolic cell-movements in animal halves led to the formation of a frill of tissue in the region of the former equator. In the experiment discussed above, which was carried out at a higher temperature, these signs of gastrulation movements were already visible at the end of the 4–6 hour incubation period. This was perhaps related to the increased uptake of phosphate in this last period.

TABLE 7

The uptake of phosphate by Xenopus *half-neurulae expressed in mean counts per minute 32P taken up in the time stated with standard error*

In Experiment I each group was divided into subgroups 1 and 2, which were incubated separately.

Experiment I (September 1953). Medium 763,000 c./m./ml. 32P at 21° C.

Time after cutting (hours)		*Dorsal half*	*No. of embryos*	*Ventral half*	*No. of embryos*	*Injured whole embryos*	*No. of embryos*	*Ratio D/V*
0–2	1	190±19	15	178±15	16	115±29	6	1·07
	2	201±15	22	201±24	21	154±31	7	1·00
2–4	1	248±41	14	125±19	13	115±28	7	1·99
	2	205±30	17	143±19	16	117±55	6	1·44
4–6	1	108±30	16	70±10	17	88±22	7	1·55
	2	130±35	14	72±11	14	132±65	6	1·81

Experiment II (March 1954). Medium 350,000 c./m./ml. 32P at 19° C.

Time after cutting (hours)	*Dorsal half*	*No. of embryos*	*Ventral half*	*No. of embryos*	*Injured whole embryos*	*No. of embryos*	*Ratio D/V*
0–2	70±6	22	66±5	23	67±7	8	1·06
2–4	37±3	25	24±3	25	16±3	5	1·54
4–6	20±3	26	5±1	26	9±2	6	4·00

Half-gastrulae. Healing of the half-gastrulae was slower, but cell-division continued normally, and by the end of 6 hours both halves, now completely healed, had an archenteron and a circular blastopore enclosing a small yolk-plug. Ventral halves kept for a further 24 hours showed no further development, but dorsal halves developed into small larvae, deficient only in the abdominal region.

The uptake of phosphate was on the whole similar in the two halves at first, but became relatively much greater in the ventral half during the third 2-hour period (Table 6).

Half-neurulae. The neural folds of the dorsal halves continued to close, but so much more slowly than in the normal embryo, that after 4 hours closure was in some cases not complete. At the end of 6 hours, however, the nerve-tube was completed, there was a sucker rudiment, and, in the somites, spindle-shaped muscle-cells were differentiating. After a few days these halves formed swimming tadpoles, deformed in belly and tail regions.

The ventral halves remained throughout the experiment as spherical nodules, in some of which sections revealed a short length of axial (tail) structures. Subsequently some of these fragments formed more or less perfect tails, while others remained undifferentiated.

The uptake of phosphate was at first the same in the two kinds of fragments, but fell off much more rapidly in ventral than in dorsal halves (Table 7).

The effect of p-*dinitrophenol on phosphate uptake.* It has been shown that *p*-dinitrophenol (DNP), which is said to uncouple phosphorylation from oxidation, has a strongly inhibiting effect on amphibian embryos at concentrations of 10^{-4} M to 10^{-5} M (Brachet, 1952). *Xenopus* embryos were found to respond to 10^{-4} M DNP in a manner similar to that of the other Amphibia studied. Development was at first retarded, but even after 4 hours, when the embryos had become stained yellow, recovery was complete a few hours after return to normal culture conditions. After longer periods in DNP recovery was not possible.

TABLE 8

The effect of dinitrophenol on the rate of uptake of phosphate by fragments of Xenopus *embryos expressed in mean counts per minute 32P per embryo with standard error*

The numbers in brackets represent the number of fragments in each group.

Neurulae

		Dorsal half		*Ventral half*	
Temp. (° C.)	*Time after cutting (hours)*	*DNP*	*Control*	*DNP*	*Control*
19°	0–2	15±1	70±6	19±1	66±5
		(23)	(22)	(23)	(23)
	2–4	12±1	37±3	12±2	24±3
		(21)	(25)	(20)	(25)
	4–6	13±2	20±3	4±1	5±1
		(19)	(26)	(18)	(26)

Blastulae

		Animal half		*Vegetative half*	
Temp. (° C.)	*Time after cutting (hours)*	*DNP*	*Control*	*DNP*	*Control*
19°	0–2	9±1	20±2	13±1	17±1
		(21)	(20)	(17)	(14)
	2–4	2±1	5·5±1	5±1	8±1
		(24)	(23)	(23)	(20)
	4–6	3±1	8±2	6±0·1	9±2
		(29)	(21)	(19)	(14)

Comparison of phosphate uptake by whole embryos in the presence and in the absence of DNP gives somewhat equivocal results, and would appear to depend on the degree of cytolysis. One batch of neurulae showed only a slight difference: controls took up 1·8 ± 0·2 counts per minute of 32P in 2 hours, DNP-treated embryos 1·3 ± 0·2 counts per minute (five groups of embryos in each case). In both groups the neural folds closed during the incubation. A group of gastrulae, incubated at the dorsal lip stage, showed a greater effect of the DNP: controls, 8·3 ± 1·3 counts per minute 32P (six groups), DNP-treated 1·5 ± 0·2 counts per minute (seven groups). At the end of the 2-hour incubation period the controls had reached the small yolk-plug stage, the DNP-treated from large to medium yolk-plug stage. Another batch of embryos was divided into three groups, one of which was pretreated for 2 hours with 10^{-4} M DNP solution before incubation in the DNP solution which contained 32P. Development in the treated embryos was retarded more drastically than in the previous batch. The results were as follows: controls, 1·5 ± 0·2 counts per minute 32P taken up; in presence of DNP, 3·3 ± 0·9 counts per minute; pretreated with DNP, 3·0 ± 0·5 counts per minute (about fifteen embryos in each group).

After prolonged immersion, when cytolysis was more advanced, the DNP-treated embryos took up much more phosphate than the controls—presumably by adsorption on the dead cells. After 15 hours in 32P medium controls had taken up 12 ± 1 counts per minute (10 separate embryos), those cultured in DNP/32P solution had taken up 393 ± 18 counts per minute (eight separate embryos).

The effect of DNP on phosphate uptake by fragments is much more marked, as shown in Table 8. The DNP did not prevent or retard the healing of the cut surfaces. The DNP-treated blastula-halves formed epibolic frills and groove-like blastopores in the same way as the controls. However, elongation of the dorsal halves of neurulae was prevented by the DNP. These results were confirmed by further experiments.

DISCUSSION

In the first place it must be decided whether the entry of phosphate into these embryos can be considered as a metabolic function, or whether it is incidental to a flow of the medium into internal spaces, directly into the archenteron, or through the wound in injured embryos, or indirectly into the blastocoel, e.g. between the cells. Measurements of the swelling of *Xenopus* embryos during development were made in relation to this point. The diameter of each of about ten embryos was measured at intervals under the microscope with an eyepiece micrometer scale. The volume of the embryo was then calculated. The measurements were repeated on several other batches of eggs, which gave similar results.

It was calculated from these results that the average volume increase from the 32-cell stage to stage 10 (dorsal lip of blastopore) would be 0·036 cu. mm. per 120 minutes, that from stage 10 to stage 12 (beginning of neurulation) would be

0·045 cu. mm. If the data presented in Table 1 are now considered, it can be seen that in experiments 2 to 5, these volumes of the medium would contain from 48 to 57 counts per minute for the cleavage and blastula stages, and from 60 to 71 counts per minute for the gastrulation stages. These values are considerably in excess of the actual uptake, so that during imbibition of water a part, usually most, of its phosphate content is excluded.

Injured embryos and fragments, on the other hand, take up much *more* 32P than can be accounted for by influx of the medium, so that this also is evidence of selective behaviour by the cells.

A striking effect of fertilization upon the penetration of phosphate was found by several workers in a number of echinoderm species (Brooks & Chambers, 1948; Lindberg, 1948, 1950; Abelson, 1947; Marshak & Harting, 1948; Chambers *et al.*, 1948; and Whiteley, 1949). Brooks & Chambers (1954) later showed in two species of *Strongylocentrotus* that there was in fact a time lag of 15–20 minutes after fertilization before the 32P uptake began to increase, and the maximum was only reached after 50–90 minutes. In the effect of fertilization on phosphate uptake, therefore, the echinoderms resemble the axolotl.

In general it has been shown that after this increase at fertilization the rate of phosphate uptake remains constant in echinoderm embryos (e.g. Villee *et al.*, 1949), with possibly a 'mitotic rhythm' in some species (Zeuthen, 1951) though not in all (Whiteley, 1949). As in the case of Amphibia, the isotope was not usually lost once it had been taken up (except by unfertilized eggs, Brooks & Chambers, 1954). Uptake of phosphate by the echinoderm embryos was greatly reduced but not completely prevented by such inhibitors as 4–6 dinitro-*o*-cresol and usnic acid (Abelson, 1947; Marshak & Harting, 1948). These effects were much more striking than the effects of DNP on the penetration of phosphate into the amphibian embryos.

In these respects it can be seen that the obviously functional phosphate intake of echinoderm embryos is more susceptible to changes in outside conditions and metabolic interference than the 'vestigial' phosphate intake or exchange of *Xenopus* embryos.

The phosphate uptake of echinoderm embryos appeared in some species to be correlated with oxygen uptake (Brooks & Chambers, 1954) but this is clearly not so in Amphibia. Brachet (1954) found no increase in oxygen uptake at fertilization (*Rana temporaria* = *fusca*) although the R.Q. dropped from 0·99 to 0·66. The curve obtained by Tuft (1952) for oxygen uptake by *Xenopus* embryo does not resemble the phosphate uptake curve. Similarly, the results for fragments of embryos do not show any correlation with what is known of differential oxygen uptake in dissected parts of amphibian embryos. For instance, Brachet (1947) and Boell (1948) reviewed experiments showing a similar rate of uptake of oxygen by dorsal and ventral fragments of gastrulae, in contrast to the results for phosphate uptake. Consumption of oxygen was, however, found to be higher at the animal than at the vegetative pole. If there were a correlation between oxygen uptake and phosphate absorption, therefore, the animal halves of blastulae might be expected to take up more phosphate than the vegetative halves towards the end of the 6-hour period studied, when gastrulation movements begin; but this is not so.

It remains possible that differences in phosphate uptake may be related to differences in synthetic activity of the cells. It has been shown in many different ways that synthetic activity becomes important at the beginning of gastrulation (Brachet, 1952) and this is the stage at which most phosphate is taken up, especially by fragments. Kutsky (1950) also showed that while the distribution of introduced 32P among phosphate compounds of *R. pipiens* embryos was unchanged during cleavage, there was at gastrulation a shift of 32P from acid-soluble to acid-insoluble fractions, indicating synthetic activity involving phosphate at this stage. The drop in phosphate uptake at the tail-bud stage, when differential synthesis is proceeding, may be a consequence of the differentiation of the epidermal cells as a more effective barrier, Mezger-Freed (1953) also showed a decrease in phospho-protein phosphatase activity after closure of the neural tube (*R. pipiens*). There is, however, no immediate correlation with alkaline phosphatase activity, which rises steadily during the development of *Xenopus* (Krugelis, 1950). The dorsal halves of neurulae may be considered as showing an adaptive increase in phosphate uptake, deprived as they are of their yolk, the main source of larval phosphate.

In fragments of gastrulae, however, it is the dorsal half in which differentiation is most rapidly proceeding, and yet it is the ventral half which, towards the end of the 6 hours, takes up more phosphate. Grant (1954) found no significant differences between distribution of 32P in dorsal and ventral halves of *R. pipiens* gastrulae labelled as ovarian eggs. Regional differences in respect of phosphate metabolism clearly require further investigation. Experiments are at present being carried out to determine into which organic compounds the introduced 32P is incorporated. Preliminary results suggest that from about 10 to 20 per cent. of the phosphate incorporated into the fragments here studied was in acid-insoluble form, and that almost all of the 32P was contained in the liquid phase of the cytoplasm and in the small granules (microsomes).

The results for phosphate uptake by fragments show some resemblances to uptake of amino-acids as reported by Friedberg & Eakin (1949) and Eakin *et al.* (1951) especially at the neurula stage, when glycine was much more readily incorporated into dorsal halves than into ventral. Dorsal halves of gastrulae of *R. pipiens* and *Hyla regilla* took up the same amount of glycine as ventral halves, and a little more methionine. Unfortunately the results were not analysed into an initial healing and subsequent differentiating periods, so that differences which were only apparent in the experiments here reported during the 4–6 hour period after cutting could not be expected to appear.

Finally, the curious results obtained with microcephalic embryos need further investigation. The great difference in 32P uptake by these abnormal embryos indicates some disturbance in phosphate metabolism. Gustafson & Hasselberg (1951) suggested, as the result of work on the enzyme systems of developing sea-urchins, that lithium (which also causes microcephaly in Amphibia) may act as a 'vegetalising agent' by an effect on the phosphorylating system. This seems to offer a convenient means of studying the effects of lithium on amphibian embryos, if it should prove that artificially produced microcephalics also take up an excessive quantity of phosphate. Work along these lines is already in progress, and so far indicates that *Xenopus* embryos do in fact take up more phosphate in the presence of lithium.

In this connexion it is interesting that Brachet (1954), using frogs, found that hybridization and treatment of the sperm with nitrogen mustard altered the rate of phosphate uptake of the resulting embryos as compared with controls. Work of this type may be expected to elucidate eventually the role of the nucleus in the important phosphate exchanges in developing embryos.

SUMMARY

1. Embryos of *Siredon mexicanum* and *Xenopus laevis* were shown, by the use of 32P, to take up small but measurable amounts of phosphate from the culture medium.

2. The rate of uptake was higher in embryos than in unfertilized eggs, and was related to the stage of development. The rate was proportional to the concentration of phosphate in the medium.

3. Spontaneously occurring microcephalic embryos took up much more phosphate than normal embryos.

4. Injured embryos and dissected fragments of embryos took up more phosphate than intact embryos. Dissected neurulae took up more phosphate than dissected blastulae, and half-gastrulae took up more than either.

5. Vegetative fragments of blastulae took up more phosphate than animal fragments, particularly towards the end of the 6-hour period studied.

6. Ventral fragments of gastrulae took up the same amount of phosphate as dorsal halves during the first 2 hours after cutting, but subsequently took up more.

7. The phosphate uptake of dorsal fragments of neurulae was at first the same as that of ventral fragments, but from 4 to 6 hours after cutting was about twice as rapid.

8. 10^{-4} M *p*-dinitrophenol had a slight inhibiting effect on uptake of phosphate by whole embryos, counterbalanced by adsorption of 32P on killed cells. The phosphate uptake of fragments was cut down by the inhibitor to about one-half to one-third the level of the controls.

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Long-term Changes in the Size and Collagen Content of Scars in the Skin of Rats

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INTRODUCTION

A PREVIOUS paper (Abercrombie, Flint, & James, 1954) described the early changes in size and collagen content of the granulation tissue and subsequent scar that fill a small wound made in the dorsal skin of a rat. The observations were carried up to the 25th day after wounding. It was found that by about the 10th day the wound had contracted down to a minimal area and wet weight, and that it remained at or near this minimum during the remaining 15 days of observation. The collagen content of the repair tissue increased during the phase of contraction and continued to do so in the completely contracted wound. In the present paper we describe the later evolution of the scar up to 300 days after wounding. The reduced area and wet weight reached by 10 days have proved to be merely temporary minima. A phase of growth of the scar follows. Our previous suggestion that collagen formation may continue beyond 15 days after wounding turns out to be correct, but something of an understatement: at least ten times as much collagen is formed after 15 days as before.

MATERIAL AND METHOD

Animals

Adult male white rats were used, of a mean body-weight of 243·7 g., range 182–321, standard deviation 35·1. They were divided into five treatment groups, differing in length of time (15, 50, 100, 200, or 300 days) between wounding and autopsy. Tattooing and operation were performed on fourteen batches each of five rats. Each batch contributed to two different treatment groups. The number of animals finally available for analysis in each treatment group was as follows: at 15 days, 10; at 50 days, 8; at 100 days, 13; at 200 days, 10; and at 300 days, 10. Body-weight at the time of operation did not differ significantly between the treatment groups (the variance between groups being less than the variance within groups). Mean body-weight gains during the experiment were: 15-day

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group 36 ± 3; 50-day group 103 ± 8; 100-day group 176 ± 9; 200-day group 118 ± 26; and 300-day group 205 ± 20. The 200-day group is anomalous because it contained four members of one batch the whole of which failed almost completely to grow.

Tattooing and operation

Techniques were much the same as those used previously (Abercrombie, Flint, & James, 1954). A machine with eight needles was, however, used to make a tattoo pattern of eight dots, describing a square of approximately 25–30 mm.^{2} in area (measured through the dot centres) in the skin to one side of the midline of the dorsolumbar region. The marks were driven by the needles almost down to the deep fascia. Within this tattoo pattern bordered by the dots, a square about 20 mm.^{2} in area of the full thickness of the skin was excised. In the 100, 200, and 300-day groups a second, control square, which remained unwounded, was tattooed on the other side of the dorsal midline, so that the normal growth in area of the skin could be assessed. The size and disposition of the tattoo marks were reproduced on tracing-paper before operation and before autopsy. In the 100 and 300-day groups tracings were also made at intermediate times, enabling us to follow the evolution in area of individual wounds and control areas. The areas marked out by the tattoos were measured from the tracings by the photoelectric method as previously described. Excision, both during the initial wounding and during the final removal of the scar and control area at autopsy, was made within the inner margins of the tattoo dots, after the skin had been treated with cedar-wood oil to make the dots as clear as possible. The margin of normal skin, which was usually apparent, was an additional guide to the excision of the scars. It is difficult to excise the scar accurately within the eight dots when it is at its minimum size, so that the 15-day group is doubtless less reliable than the later groups. The excised pieces—the initial piece of normal skin removed at operation, and the scar and control piece of normal skin removed at autopsy—

TABLE 1

Mean area, wet weight, and hydroxyproline content (with standard errors) of the pieces of normal skin removed at initial operation in the five groups of rats killed at different intervals of time afterwards

N = number of animals, HYP = hydroxyproline

(1) Group	(2) *N*	(3) *Area* (*mm.*^{2})	(4) *Wet weight* (*mg.*)	(5) *Weight/area*	(6) *HYP* (*μg.*)	(7) *HYP/area*	(8) *HYP/ weight*
15 day	10	16·3±0·6	20·2±1·6	1·25±0·10	591±32	36·8±2·6	30·9±2·8
50 day	8	15·9±0·6	22·4±2·4	1·45±0·22	604±36	38·9±3·7	30·3±4·0
100 day	13	20·8±0·8	27·5±2·0	1·33±0·09	640±37	31·2±1·9	24·2±1·4
200 day	10	17·1±0·5	22·7±1·9	1·33±0·12	607±37	35·6±2·2	27·6±1·7
300 day	10	22·7±1·5	32·3±2·7	1·46±0·11	720±35	32·6±2·2	23·5±1·9

were weighed wet, and their collagen was estimated by the hydroxyproline technique of Neuman & Logan (1950). Results of the latter estimation are given in microgrammes of hydroxyproline.

The degree of standardization attained in making the initial wounds is shown by the area, weight, and hydroxyproline content of the pieces removed (Table 1). It is evident that the different treatment groups are not homogeneous. The 15, 50, and 200-day groups are fully comparable with each other; but, because of a temporary defect in the spacing of the tattooing needles, the 100 and 300-day groups, though they do not differ significantly from each other, differ from the other three groups in that they have received on the average larger wounds. This failure of randomization must be taken into account in assessing the results. On the other hand, within each of the groups, the area of skin removed at the operation did not differ significantly from the corresponding control areas at that time. The mean area (in mm.^{2}) within the tattoos in the 100-day group was $20{\cdot}8 \pm 0{\cdot}8$ and $20{\cdot}4 \pm 1{\cdot}1$ on repair and control sides respectively; in the 200-day group $17{\cdot}1 \pm 0{\cdot}5$ and $16{\cdot}3 \pm 0{\cdot}9$; and in the 300-day group $22{\cdot}7 \pm 1{\cdot}5$ and $22{\cdot}2 \pm 0{\cdot}9$.

The composition of the skin at the time of the operation did not differ between the groups: wet weight or hydroxyproline content when standardized for area by analysis of covariance, and hydroxyproline content when similarly standardized for wet weight, do not differ significantly between the groups ($P > 0{\cdot}2$).

Stability of tattoos

The tattoo marks were not the only guide to measurement and excision of a scar, since the boundary between normal skin and scar could usually be seen. We depended, however, entirely on the tattoo marks for determining the amount of control skin to be excised. We assume therefore that the marks have adequate stability; and the long periods of time make it necessary to examine whether we can rely on this. During the experiment each dot enlarged slowly in surface area, as is to be expected from the general growth of the skin. This general growth can be assessed from the growth of the area enclosed by a line drawn through the centres of the dots: an area which should be unaffected by independent changes in dot size. The actual change in dot size proved to be larger in some groups and smaller in others than was to be expected from the general growth of the skin. The discrepancy, though consistent within any one group at successive times, did not amount to more than 10 per cent. of the area demarcated by the dot centres. Change in either direction is probably due to dispersion of the carbon particles, which can be seen to have occurred in sections: a little dispersion spreads the dot; much dispersion dilutes its periphery so that it appears to shrink. The same methods of estimating the instability of the marks cannot be applied to the tattoo marks at the edge of the wound, because it is not known what allowance should be made here for growth of the skin within which the marks are embedded.

We have not attempted to follow quantitatively the vertical distribution of the tattoo marks. In sections of marks in unwounded skin and during the first 25 days after wounding the marks extend almost or quite through to the subcutaneous tissue beneath the panniculus carnosus. At the 100th day after wounding the depth of the marks is approximately the same in sections. By gross inspection during excision it is still the same at the 300th day. Throughout the period the marks seemed to remain substantially vertical to the skin surface.

RESULTS

Area of scar

It was obvious by simple inspection that by the 15th day after operation the wounds had contracted, assuming the usual shape of a 4-pointed star (Abercrombie, Flint, & James, 1954); and that they subsequently expanded again, without however reverting towards their original square shape. The control areas equally obviously had grown throughout the period of observation.

These growth changes are expressed in Table 2 by measurements of the area enclosed by the dot centres. Any changes in dot size, provided they take place symmetrically, which they seem to do, are thereby made irrelevant. The disadvantage of this method of measurement, when applied to the wounded side, is that it does not express changes in size of the scar alone, but of the scar together with a surrounding rim of original skin, representing the radius of the dots, which contributes significantly to the total area when the scar is small. Such measurement are best made successively on the same animals, to avoid superimposing variation between animals on variation between times. We therefore quote only mean tattoo areas of the members of the 100-day group (group A) and of the 300-day group (group B), which were measured at intervals: the 100-day group at 15, 30, 60, and 100 days; the 300-day group at 15, 50, 100, and 300 days. There is progressive growth of the control tattoo area. After the very sharp contraction of the wounded tattoo area, there is an expansion, rapid at first, then slower. Since growth-curves based on means have undergone a statistical smoothing out, which may disguise the real course of events during the growth of an individual,

TABLE 2

Mean tattoo areas (measured through centres of tattooed dots), with standard errors, in two groups of rats (A, 9 rats; B, 10 rats) each rat bearing a wounded and a control area; measured at successive times after wounding

		Days after wounding						
Group		*0*	*15*	*30*	*50*	*60*	*100*	*300*
Wound	A	30·7±1·1	13·5±0·5	17·3±1·0	—	21·6±1·4	23·3±0·8	—
	B	33·6±1·4	12·4±0·8	—	20·4±1·1	—	23·0±1·3	28·9±1·9
Control	A	30·3±2·2	34·2±2·4	38·3±3·0	—	41·0±3·3	46·5±2·5	—
	B	33·5±1·3	34·5±1·9	—	41·3±2·5	—	44·3±2·5	52·9±3·0

we should add that the size changes of the individual scars follow the same pattern.

There are changes in amount of growth per day during the period of scar expansion. When the two groups are combined, the growth per day of the tattooed area of the wounded side between 15 and 50–60 days is 0·202 ± 0·015 mm.^{2}; between 50–60 and 100 days it is 0·048 ± 0·011 mm.^{2}; and (in the 300-day group only) between 100 and 300 days it is 0·029 ± 0·005 mm.^{2} Measurements of the areas within the inner borders of the tattoo points, representing the size of the scar itself qualified by errors due to change of dot size, also follow the same pattern: growth per day between 15 and 50–60 days is 0·134 ± 0·011 mm.^{2}; between 50–60 and 100 days it is 0·044 ± 0·009 mm.^{2}; and between 100 and 300 days it is 0·017 ± 0·044 mm.^{2} There is evidently therefore a well-marked decline with time in the growth rate of the scar. There is a similar decline in growth per day of the control area, which between 15 and 50–60 days is 0·170 ± 0·027 mm.^{2}; between 50–60 and 100 days 0·094 ± 0·025 mm.^{2}; and between 100 and 300 days 0·043 and 0·008 mm.^{2}

The absolute amount of new area added per day is rather similar whether the tattoo encloses a scar or an area of normal skin. Over the whole of the first 100 days after wounding the addition of new area is negligibly different between wounded and control sides (10·3 ± 0·7 mm.^{2} and 11·1 ± 1·3 mm.^{2} respectively). But though the means are so similar, the wounded side is less variable than the control: the variances are indeed significantly different ($F = 3{\cdot}45$, d.f. 18 and 18, $0{\cdot}05 > P > 0{\cdot}01$). The similarity of the wounded and control sides in the absolute addition of new area implies a great difference in growth *rate*, since the area on the wounded side has been initially much reduced by contraction. In terms of specific growth rate, the wounded area grows highly significantly faster than the control up to 50 to 60 days, but thereafter there is no significant difference.

Shape of scar

The original tattoo mark describes a square with two sides parallel to the long axis of the animal. Contraction of the wound converts the square into a symmetrical 4-pointed star as described by Abercrombie, Flint, & James (1954), but the set of corner dots and the set of mid-side dots continue each to mark out an approximate square. The contraction (during the first 15 days) occurring between the corner dots in the direction along the length of the animal does not differ significantly from the contraction transverse to the length of the animal: the difference between these contractions is 0·17 ± 0·16 mm. The subsequent expansion of the scar is, however, slightly asymmetrical. The longitudinal expansion (between corner dots, during the period of 15 to 100 days) is greater than the transverse expansion. The difference between them (0·35 ± 0·16 mm.) is only of border-line significance (t 2·17, d.f. 19, $P = 0{\cdot}05$); but if the expansion is expressed as relative to the length of side at 15 days, the difference becomes highly significant (t 3·26, d.f. 19, $P < 0{\cdot}01$). Furthermore, the degree of stellateness is slightly reduced in the longitudinal direction, the distance between the mid-side points increasing rather more than that between the corner points during the period from 15 to 100 days; while in the transverse direction the degree of stellateness slightly increases. The difference is just significant at the 5 per cent. level.

The control area has no tendency to take up a stellate form but it does not remain a square. During the period of contraction of the wound it becomes elongated towards the wound. Between 0 and 5 days its length in the longitudinal axis of the animal diminishes, and its length in the transverse axis increases, the difference between the changes in the two axes (0·52 ± 0·18 mm.) being significant (t 2·94, d.f. 19, $P < 0·01$). Between 15 and 100 days this initial distortion is a little reduced: like the scar, the control area grows somewhat more in the longitudinal than in the transverse direction. Unlike the scar, the difference between growth in the two directions is not significant, perhaps because the linear growth of the control skin is in absolute terms less than that of the scar, and hence more obscured by errors of observation. We have investigated whether asymmetry of scar growth is correlated with asymmetry in control area growth within individual animals. Using the growth between 15 and 100 days, relative to the length of side at 15 days, and expressing the asymmetry as before by subtracting the growth in the longitudinal direction from that in the transverse direction, we obtained a correlation coefficient between scar and control area of +0·431 (d.f. 18), which is almost significant at the 5 per cent. level. There is here, therefore, a suggestion of a common response of scar and normal skin to factors influencing the pattern of growth.

Wet weight

There are greater uncertainties about the analysis of the wet-weight changes of the scar than of its area changes. This is partly because of the greater technical difficulty of measuring wet weight; and partly because we are obviously unable to follow wet weight in the same group of rats through the time course of repair but must use a succession of different groups of rats.

TABLE 3

Measurements (means and standard errors) made on scars
N = number of animals, HYP = hydroxyproline

(1) *Period of repair, days*	(2) *N*	(3) *Area (mm.²)*	(4) *Wet weight (mg.)*	(5) *weight/area*	(6) *HYP (µg.)*	(7) *HYP/area*	(8) *HYP/weight*
15	10	5·5±0·4	4·2±0·6	1·08±0·25	77±8	14·6±1·6	19·6±2·4
50	8	9·5±0·6	13·6±0·8	1·49±0·17	395±23	43·2±4·9	26·6±0·7
100	13	13·2±0·6	14·7±1·6	1·12±0·11	515±42	39·5±3·4	37·3±2·9
200	10	12·7±0·9	18·4±3·4	1·38±0·15	748±137	56·4±5·9	41·7±2·8
300	11	16·4±1·1	20·7±1·6	1·26±0·04	903±64	55·9±3·5	43·7±2·6

The mean weights of the excised scars are given in Table 3, col. 4. There is evidently a sharp rise in weight between 15 and 50 days after wounding, and then a suggestion of an upward trend continuing to the end of the longest period observed. As mentioned in the section on Method, however, the different groups were not satisfactorily standardized for the size of the initial wound. Adjustment of the mean weights by analysis of covariance to allow for the variation in initial wound area does not, however, seriously alter the picture. The adjusted means for the five successive time groups are respectively 4·9, 14·4, 14·3, 18·8, and 19·8. We have tested whether there is a significant rise in weight in the period from 50 to 300 days by regressing the wet weight at autopsy against time, using multiple regression analysis to remove any effects of the non-homogeneity of initial area. The partial regression coefficient in microgrammes of wet weight per day is 27·8 ± 11·8 ($t = 2{\cdot}35$, d.f. 39, $0{\cdot}05 > P > 0{\cdot}02$). The relation with initial wound area is not significant. The areas of the scars are also given in Table 3 (col. 3), and it is evident that the growth in weight corresponds to the growth in area. Weight per unit area (col. 5) does not change significantly with time.

The uninjured skin on the control side grows in area, as already discussed, and in wet weight also (compare the initial weight of the skin within the tattoo mark on the operated side, Table 1, col. 4, with the final weight on the control side, Table 4, col. 4). It furthermore appears that in normal skin there is a slight increase with time in weight per unit area. This only emerges significantly if we standardize the data by taking for each rat the difference between weight per unit area at initial operation and weight per unit area at autopsy. Pooling the data of the 100-, 200-, and 300-day groups the increase (0·22 ± 0·09) is just significant ($t = 2{\cdot}31$, d.f. 32, $0{\cdot}05 > P > 0{\cdot}02$). Evidently thickening of the skin with age is much less well marked than is growth in area. The weight per unit area of the scar (Table 3, col. 5) is significantly less (at the 1 per cent. level) than that of the normal skin (Table 4, col. 5) of the same animals, taking the 100-, 200-, and 300-day groups separately. Exactly comparable normal skin is not available for the 15 and 50-day groups.

TABLE 4

Measurements (means and standard errors) made on control areas of normal skin in three groups of rats. The areas were marked at the beginning of the experiment and removed at the time of excision of the scars

N = number of animals, HYP = hydroxyproline

(1) Group	(2) *N*	(3) *Area* (*mm.*^{2})	(4) *Wet weight* (*mg.*)	(5) *Weight/area*	(6) *HYP* (*μg.*)	(7) *HYP/area*	(8) *HYP/weight*
100 day	13	29·2±1·5	43·1±2·6	1·48±0·06	1140±50	39·6±1·7	27·0±1·1
200 day	10	24·8±2·6	40·9±5·8	1·63±0·16	1120±145	44·6±2·7	28·0±1·0
300 day	11	37·7±2·2	62·1±4·6	1·66±0·10	1680±99	45·1±2·4	27·7±1·3

Hydroxyproline content

Like the measurements of wet weight, those of hydroxyproline content are relatively less efficient than are the area measurements because the time-course has to be constructed from successive groups of animals. Table 3 (col. 6) gives the mean total amount of hydroxyproline in the scar at different times after wounding. There appears to be a sharp rise in hydroxyproline content between 15 and 50 days, followed by a steady upward trend. Adjusting the means by covariance analysis to remove the effects of differences in initial wound-size does not alter this picture: the means of the five successive times groups become 90, 409, 505, 765, and 885 respectively. The difference between any successive two of these means, apart from that between the 15- and 50-day means, is not significant or is only just significant at the 5 per cent. level. When, however, the change in hydroxyproline content between 50 and 300 days is analysed by multiple regression, with time since wounding and initial wound size as variables, the regression on time is highly significant (the partial regression coefficient, in microgrammes hydroxyproline per day, is $2{\cdot}00 \pm 0{\cdot}44$, $t = 4{\cdot}52$, d.f. 39, $P < 0{\cdot}001$). The regression on initial wound size is not significant. We may conclude that collagen is laid down during most or all of the time covered by our experiments. In earlier work (Abercrombie, Flint, & James, 1954) we found the mean hydroxyproline content of similar wounds at 15 days to be 143 ± 23 μg., almost twice the value now obtained. The discrepancy is undoubtedly due to the use of 4-point tattoos for the earlier paper, which, as there discussed, leads to the inclusion of serious amounts of normal skin.

The increase of hydroxyproline content with time is accompanied, as we have already shown, by an increase in area and wet weight of the scar. The formation of hydroxyproline proceeds rather faster, however, than does weight and area increase. There is an increase of hydroxyproline per unit area (Table 3, col. 7), which is obvious between 15 and 50 days, and can be shown between 50 and 300 days by regression against time (the regression coefficient in μg. hydroxyproline per mm.^{2} per 100 days is $7{\cdot}05 \pm 2{\cdot}36$, $t = 2{\cdot}98$, d.f. 40, $0{\cdot}01 > P > 0{\cdot}001$). Since it has been shown that weight per unit area does not change significantly with time, it is not surprising to find that the concentration of hydroxyproline per unit weight (Table 3, col. 8) increases significantly with time. There is no need to test the obvious significance of the increase between 15 and 50 days. Between 50 and 300 days regression against time shows a significant rise (the regression coefficient in μg. hydroxyproline per mg. per 100 days is $5{\cdot}33 \pm 1{\cdot}41$, $t = 3{\cdot}8$, d.f. 40, $P < 0{\cdot}001$).

Evidently (col. 6 of Tables 1 and 3) at some time between 100 and 200 days the collagen originally removed has been replaced. During this period, however, a comparable area of normal skin has been growing not only in area and wet weight but also in total hydroxyproline content (compare Table 4, col. 6 with Table 1, col. 6). In normal skin, as in the scar, the laying down of collagen proceeds faster than the growth of area, so that the hydroxyproline content per unit area increases (Table 4, col. 7). The increase is not as clear as it is in the scar: the regression coefficient of hydroxyproline per unit area against time is not quite significant at the 5 per cent. level. The normal skin at the end of the experiment in the 100-, 200-, and 300-day groups of rats has, however, in each group significantly more hydroxyproline per unit area (Table 4, col. 7) than the corresponding samples of normal skin taken from the same three groups of rats at the beginning of the experiment (Table 1, col. 7). For the difference within the 100-day group, $t = 3{\cdot}28$, d.f. 24, $P < 0{\cdot}01$; for the 200-day group $t = 2{\cdot}36$, d.f. 18, $0{\cdot}05 > P > 0{\cdot}02$; and for the 300-day group $t = 3{\cdot}84$, d.f. 19, $P < 0{\cdot}01$. A progressive but small increase in hydroxyproline per unit area seems the most likely interpretation. We have already shown that normal skin is probably increasing, too, in weight per unit area (presumably representing thickness), and this almost accounts for the increase in hydroxyproline per unit area; there is no sign of any change in concentration per unit of wet weight with time in the three control groups (Table 4, col. 8). When, however, the concentrations found in these three control groups are pooled and compared with the concentrations found in the same rats at the beginning of the experiment (Table 1, col. 8), a significant difference can be demonstrated ($t = 3{\cdot}10$, d.f. 32, $P < 0{\cdot}01$). The increase in hydroxyproline in relation to the weight or area of tissue available to form it is obviously much higher in the scar than in the normal skin, since the wounded area after its contraction is throughout smaller than the control area.

The final comparison to be made is between the concentrations of hydroxyproline in scar and in normal skin. The hydroxyproline per unit area or weight is obviously much less in a scar at 15 days than in the skin in which the wound was made (Tables 3 and 1, cols. 7 and 8). The lack of strict control skin taken at autopsy precludes any definite conclusions for the 50-day group but the concentration in the scar does not differ from that of the controls of the 100-, 200-, and 300-day groups; it seems likely therefore that the scar concentration has by 50 days caught up with that of normal skin. By 100 days the concentration per unit area in the scar obviously does not differ from that of the normal control skin (col. 7 of Tables 3 and 4), but the concentration per unit weight (col. 8) in the scar has surpassed that of normal skin ($t = 3{\cdot}30$, d.f. 24, $0{\cdot}01 > P > 0{\cdot}001$). By 200 days the scar concentration is higher both in terms of area and of weight; for area it is significantly so if the variance between rats is eliminated ($t = 3{\cdot}13$, d.f. 9, $0{\cdot}02 > P > 0{\cdot}01$), for weight it is obviously significantly higher. By 300 days the superiority of the hydroxyproline concentration of the scar in terms of both weight and area is clear enough to need no statistical testing.

DISCUSSION

The present work describes the evolution in size and collagen content of the scar left by a small skin wound during the period between 25 and 300 days after the injury. We do not yet know how representative the description we have presented is of skin wounds in general, so it is necessary to keep in mind the type of operation we have used. The wounds were small, square, full-thickness excisions made in a region of rather mobile skin in young adult rats, and left undressed.

Before surveying the changes in size and collagen content of the scar we must describe the background of change in the control area of normal skin during the same period of time. It is remarkable that the control area, situated 2–3 cm. from the wound, is nevertheless detectably distorted by the contraction of the latter. It is legitimate to assume that the contraction is the cause of the distortion, because no further distortion occurs when contraction ceases. Evidently a pull is exerted over an unexpectedly wide area around the wound, and there must be some caution about interpreting the control areas as fully representative of normal skin. The continuous growth in skin area no doubt reflects the continuous growth in body-weight of the rats, and the decline in the rate of growth of skin area expresses the ordinary decline of specific growth-rate with age, though the high rate in the 50 days immediately after operation may be partly a response to the distortion caused by the contraction. The growth in skin area is accompanied by a small increase in skin thickness, as indicated by changes in weight per unit area. Collagen is laid down in amounts slightly more than is required to keep pace with the growth in area and thickness so that there is a small rise in concentration per unit area and per unit of wet weight.

We turn now to consider the growth of the material filling the wound. The period up to 25 days after wounding can be divided into three phases, according to the data of Abercrombie, Flint, & James (1954). (1) Up to 5 days, granulation tissue is forming beneath the scab, the wound diminishes slightly in area, and a small amount of collagen is laid down. (2) Between 5 and 10 days rapid contraction occurs, diminishing the area by more than a half. This is a process in which the original cut edges of the wound are drawn inwards by a shrinkage of the wound content, the motive power being supplied, we have suggested (Abercrombie, Flint, & James, 1956), by the cells. Collagen formation continues during this phase. (3) Between 10 and 25 days the wound area is approximately stable. Since we have three successive measurements at 10, 15, and 25 days on each member of a group of animals, we know that this pause is not an artifact due to a statistical smoothing out of mass data. Collagen formation continues during this phase, too; but by the end of it the concentration of collagen in the contracted scar is still much below that of normal skin, whether expressed per unit of wet weight or per unit of superficial area.

Our present results show that a fourth phase then starts, and continues for as long as our observations have been carried. It is a phase of expansion of scar area, probably very rapid at first. Wet weight increases at the same pace, so there is probably no significant change in thickness of the scar. Collagen is laid down at a high rate per day during the period of rapid increase of area, though our data do not allow us to say that the rate is any different from that obtaining during the earlier phases of stationary or contracting area. It is formed in the scar at a higher rate per unit area or per unit of wet weight than it is at the same time being formed in the rest of the animal's skin, so that concentration of scar collagen increases steadily in relation to that of normal skin. It probably reaches the normal value, in terms either of wet weight or of area, by about 50 days. This accords well with the time of return of tensile strength to normal (Howes, 1954).

The increase in area and wet weight, initially so fast, diminishes in absolute amount added per day. Wet weight per unit area remains stable, in contrast to normal skin which is slowly growing in thickness. However, the scar continues at least for some time to lay down more collagen per day than an equivalent area of normal skin. By 100 days, therefore, the scar has surpassed normal skin in collagen per unit of wet weight; and by 200 days, in spite of the greater thickness of normal skin, in collagen per unit area too.

The later changes of collagen content here described merely put into quantitative terms the 'collagenization' of a scar which is qualitatively familiar to pathologists. What could not be demonstrated for certain from histological sections is now established for the rat, that these late changes in a scar involve more than mere packing together of existing collagen: they involve continuous new formation of collagen.

Abercrombie, Flint, & James (1954) pointed out that during the first 25 days after wounding the formation of collagen did not parallel the process of contraction. They were therefore led to reconsider the accepted theory that contraction is produced by newly formed collagen (Abercrombie, Flint, & James, 1956). The present work merely serves to reinforce our previous doubts. Collagen formation occurs continuously through the phases of contraction, of minimal area, and of expansion: it could hardly be less closely correlated with contraction.

A phase of scar expansion following the phase of contraction has been observed before in the course of experimental work (Carrel & Hartmann, 1916; Clark, 1919) though apparently long-term measurements of the phenomenon have not previously been made. Its interpretation, we believe, should be on the lines suggested by Billingham & Medawar (1955). They found that an island of skin left in the middle of a raw area, or a skin graft placed there, underwent a remarkable increase in size, which they suggested was a growth response to the tension set up by the process of contracture. They further suggested that the earlier reported instances of scar expansion might have the same explanation; and that the post-natal growth of normal skin may be adjusted to the size of the animal by a similar response to tension. Our observations on the changes in shape of the tattoo patterns during their expansion readily fall into place in this hypothesis. In the rat wounds that we have investigated the scar expansion is perhaps unusually conspicuous and long-continued because of the continuous growth in bulk of the whole animal, which superimposes a tension generated by growth on the tension generated by contraction. But a lesser degree of scar expansion may be of general occurrence. When contraction reduces a wound to little more than a linear scar, as it does in rabbits (Billingham & Reynolds, 1952; Billingham & Russell, 1956 *a*, *b*) subsequent expansion may be negligible, most of the adjustment to tension occurring by intussusceptive growth of the surrounding normal skin (Billingham & Medawar, 1955). But even in rabbit scars expansion may become obvious when contraction fails to go to completion (Billingham & Russell, 1956*a*).

SUMMARY

1. A description is provided of the changes in area, wet weight, and collagen content of the scar left by a small skin wound in young adult rats during the period between 15 days and 300 days after wounding. The corresponding changes in normal skin were also followed.

2. A standard square area of skin of about 20 mm.^2 was marked by tattooing on the back of each of a number of rats, averaging about 250 g. in body-weight. The area was then excised and its collagen content estimated by the hydroxyproline technique of Neuman & Logan (1950). The wound was allowed to heal without dressing. At 15, 50, 100, 200, and 300 days after wounding groups of the animals were killed, the scars measured, and their hydroxyproline content estimated. Control areas of normal skin, similarly tattooed at the same time as the wounded area, were measured in the 100-, 200-, and 300-day groups, and excised for collagen estimation at the same time as the scars.

3. After the period of contraction and the following phase of stationary area, which according to previous work lasts until about 25 days after wounding, the scar starts to expand, and probably continues to do so throughout the period of observation. The expansion is rapid at first, but diminishes in rate. An initially comparable area of normal skin also grows throughout at a diminishing rate per day, but, at least up to about 50 days after wounding, at a lower rate per unit of area already present than the scar.

4. The wet weight of the scar increases proportionately to its increase in area. The wet weight of normal skin probably increases a little faster than its area, and the wet weight per unit of area of normal skin eventually surpasses that of the scar.

5. Collagen formation in the scar probably occurs throughout the period of scar expansion, at such a rate as to increase the content per unit of area and of wet weight. Normal skin is at the same time also increasing its collagen content per unit of area and of wet weight, but not so fast as the scar. Consequently by about 50 days the scar concentration per unit of wet weight or of area has caught up with that of control skin; by 100 days the scar clearly surpasses the normal skin in collagen per unit of wet weight, and by 200 days in collagen per unit of area.

6. The growth of the scar is interpreted according to the hypothesis of Billingham & Medawar (1955), as a response to tension.

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Some Histochemical Observations on the Endometrium and the Yolk-sac Placenta of *Erinaceus europea*

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WITH THREE PLATES

INTRODUCTION

THE placentation of *Erinaceus europea* has been described by Hubrecht (1889). An account of the development and structure of the avascular and vascular yolk-sac placentae which are formed in this species has been presented (Morris, 1953). Reichert's membrane is formed in the wall of the yolk-sac and it persists to term. A further study of the yolk-sac placentae, Reichert's membrane, the decidua and the endometrium of *Erinaceus*, involving the use of several histological and histochemical techniques, is reported herein.

MATERIAL AND METHODS

The material consisted of the uteri of three hibernating females obtained in January and February, and the uteri of seven non-pregnant females caught at the beginning of the breeding season in Nottinghamshire in May 1954. Twenty-four gravid uteri and twelve uteri from non-pregnant lactating females were obtained between May and September.

At autopsy the uteri of the non-pregnant animals were removed and small portions were fixed in various fluids. The implantation swellings of the pregnant uteri were separated and similarly treated. The conceptuses from animals in mid and late pregnancy were slit open laterally and the embryos removed, whereupon the uterine wall, placenta, and attached foetal membranes were fixed in one block. Occasionally small portions of the foetal and maternal tissues were dissected from the conceptuses and fixed and embedded separately.

PAS reagents and Best's carmine method were used on material fixed in Rossman's fluid at 0° C. These techniques were performed on sections with and without preliminary exposure to saliva at 37° C. for 1 hour, to differentiate between glycogen and other carbohydrates.

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The methyl-green pyronin method for RNA was employed on material fixed in Zenker's fluid. RNA was extracted from control sections by immersion in 10 per cent. perchloric acid at 4° C. for 16 hours.

Material fixed in cold acetone and formalin fixed frozen sections were used to show alkaline phosphatase (Gomori, 1941). Turnbull blue reagents and Perl's method for ferric iron were applied to material fixed in 10 per cent. neutral formalin.

Frozen sections were cut from gelatine embedded material which had been fixed in either 10 per cent. formalin or 10 per cent. formal-calcium. Selected sections were stained with Sudan black, and the Fettrot method for neutral fat was also applied. The acid haematin method and the pyridine extraction test (Baker, 1946) were used on some material.

The above procedures were carried out on most of the non-pregnant uteri and on all the early implantation stages of pregnancy that were collected. A series of ten of the later stages of pregnancy were selected and similarly treated. The smallest uterine swelling in this series contained an embryo measuring 2 mm. (crown-rump length), and the largest an embryo measuring 51 mm.

Some material was fixed in Champy's fluid, and a little, after Champy fixation was post-osmificated at 37° C. for periods varying from 24 to 72 hours. Pieces of the yolk-sac splanchnopleur from later stages of pregnancy were fixed separately in Helly's fluid, postchromed, and stained by Kull's triple stain.

Certain of the later stages of pregnancy in which Reichert's membrane is present were specially treated. The following techniques and stains were applied to material which had been fixed in either Zenker's fixative, Bouin's fluid, or 10 per cent. formalin: Weigert's resorcin fuchsin for elastic tissue; Gomori's silver method for the impregnation of reticulum (1937); Lillie's allochrome method for connective tissues (1951); Masson's trichrome stain, orange G; and Van Geison's stain.

Frozen sections from material fixed in formalin were mounted in water or glycerogel, or mounted and dried, and were examined in polarized light. Stained and unstained sections of material which had been variously fixed and embedded in paraffin wax were similarly examined.

RESULTS

The endometrium

In the uteri of the hibernating, oestrous, and lactating animals that were examined the cytoplasm of the sub-epithelial connective tissue is almost devoid of RNA. In the uterus of the hibernating hedgehog the cytoplasm of the uterine glands and surface uterine epithelium is stained faintly pink by the methyl-green pyronin reagents, indicating that these tissues contain very little ribonucleic acid. In the oestrous uterus, and in the uterus of the lactating animal, the staining reaction of the cytoplasm of these tissues is moderate, indicating an increase in the amount of RNA present.

Stainable glycogen is entirely absent from the endometrium of the non-pregnant uterus of *Erinaceus*. In certain sections stained by the PAS method some small areas of the interglandular connective tissue in the deeper parts of the uterine mucosa stain intensely red, and this reaction is saliva resistant.

The presence of lipoidal inclusions in the endometrium was demonstrated by the use of Sudan black and osmic acid. With Sudan black a faint dusting of the distal and basal cytoplasm of the cells of the uterine glands and the uterine epithelium occurs. Very occasionally distinct small droplets of fat can be discerned, usually more easily visible in the distal cytoplasm. There is no noticeable difference in the amount of lipoidal material present in the epithelial elements of the uterus of hibernation, the oestrous uterus, and the uterus of the lactating female. The observations made on material fixed in Champy's fluid, some of which was post-osmificated, were essentially similar to those described above.

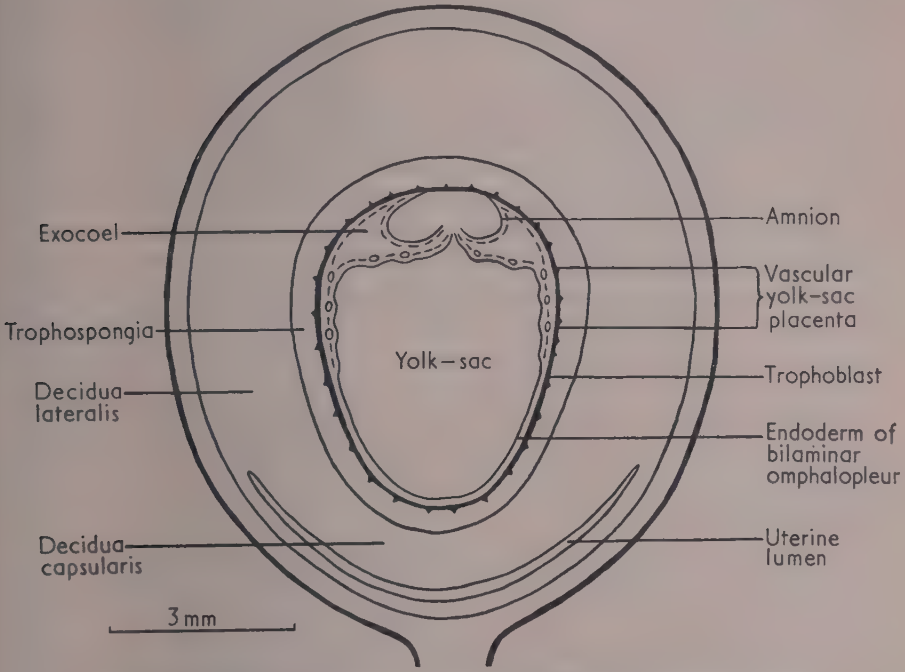
In the uterus of the hibernating hedgehog alkaline phosphatase has been observed in the cells of the uterine epithelium. It occurs mainly at the apical ends of these columnar cells. Lesser amounts are encountered in the distal cytoplasm of the epithelial cells of the necks of the uterine glands, but alkaline phosphatase is absent from the deeper coiled portions of these glands (Plate 1, fig. A). A considerable increase in the amount of alkaline phosphatase is noticeable in these epithelial elements of the endometrium of the non-pregnant oestrous uterus, and it is also present in large amounts in the apical cytoplasm and in the lumina of the deeper coiled portions of the uterine glands (Plate 1, fig. B). In the uterus of the lactating animal alkaline phosphatase is present in amounts comparable to that of the oestrus uterus, and its distribution is essentially similar.

No positive reactions for iron were obtained in the uterus of hibernation, nor in the oestrus uterus of the hedgehog. However, in transverse sections of the non-pregnant uteri of two animals which had only recently given birth to their first litters of the season, iron was found to be present in a crescentic zone on the anti-mesometrial side of the uterus in the inter-glandular connective tissue adjacent to the inner muscle tissue. No positive reactions were obtained in the uterine glands of these forms.

The early yolk-sac placenta and decidua

In the pregnant uterus the tissues adjacent to the interstitially implanting blastocyst become rapidly converted into decidual tissue of a distinct kind, the trophospongia (Hubrecht, 1889). The ducts of the uterine glands become occluded, but the epithelial elements of their deeper coiled portions can still be discerned. Early in development a decidua capsularis is formed which separates the blastocyst from the uterine lumen. In transverse sections the uterine lumen appears as a narrow crescentic cavity (Text-fig. 1).

In the early stages of pregnancy the cytoplasm of the endoderm and trophoblast, and of the adjacent trophospongia, is particularly rich in RNA. The enlarging endothelial cells of the maternal vessels present in the decidual tissue adjacent to the blastocyst also give an intense reaction. These enlarging endothelial cells contribute to the formation of the trophospongia, a compact zone of tissue surrounding the blastocyst. Elsewhere the cytoplasm of the decidual tissue appears to contain little RNA: in fact it tends to be slightly eosinophilic. The distribution of RNA remains essentially the same in all the implantation stages which were available for study—these ranged from a stage in which the embryonic knob was forming to late embryonic plate stages in which mesoderm had differentiated and amniogenesis commenced.



TEXT-FIG. 1. Diagram of a transverse section of the uterus of *Erinaceus* showing the morphological relations of the embryonic membranes and uterine tissues during amniogenesis.

Glycogen is entirely absent from the foetal tissues of the implanting blastocyst. In transverse sections of the earliest stages examined, which had been treated with PAS reagents or stained with Best's carmine, glycogen is mainly located in a circular zone around the blastocyst, comprising some of the outer trophospongia and its adjacent decidual tissue. Lesser amounts are present in the deeper decidua. In such stages the decidua capsularis appears to be formed mainly of inner trophospongia and is generally lacking in glycogen. Glycogen is absent from the epithelial elements of the deeper coiled portions of the uterine glands, but some saliva resistant and PAS positive material is present in the apical cytoplasm of some of the cells of the uterine epithelium.

In later embryonic plate stages, in which mesoderm has been differentiated and the blastocyst has enlarged considerably, the distribution of glycogen in the decidual tissues is as described above, but the staining reaction is much more intense, and glycogen in quantity is now present in the inner region of the trophospongia. The enlarging endothelial cells of the maternal vessels in the decidua appear to be devoid of glycogen.

Lipoidal material is equally abundant in the trophoblast and inner trophospongia of early and late implantation stages. Here it occurs in most of the cells as irregularly shaped dense clusters of moderately sized droplets. This material, which is absent from the endodermal epithelial cells of the bilaminar wall of the yolk-sac, is irregularly arranged within the cells, and is not indicative of any special polarity (Plate 2, fig. F). In the cells of the outer trophospongia lipoidal material is less abundant, and some scattered droplets are present in the cytoplasm of the cells of the adjacent decidual tissue. Lipoidal droplets are absent from the enlarging maternal endothelial cells, and there is no noticeable increase in the amount of sudanophil material in the epithelial elements of the deeper coiled portions of the uterine glands. The Fettrot method reveals that almost all of this material is neutral fat, but a little acid haematin positive material is present in the trophoblast and trophospongia, indicative that some phospholipid is present in these tissues.

In the earliest stage of implantation in which alkaline phosphatase was shown by Gomori's calcium-cobalt method, the blastocyst measured 0·25 mm. across its greatest diameter. At this stage the embryonic knob is beginning to form. Only very slight traces of alkaline phosphatase were found in the cytoplasm of the endoderm, trophoblast, and trophospongia—at this stage the latter is in an early phase of its development. However, some alkaline phosphatase is present in the nuclei—particularly the nucleoli—of the endoderm and trophoblast (Plate 1, fig. C). In early embryonic plate stages the amount of alkaline phosphatase in the cytoplasm of these tissues has increased (Plate 1, fig. D). In later stages, when the mesoderm is being differentiated, the endoderm and trophoblast of the blastocyst are particularly rich in alkaline phosphatase, but the reaction obtained in the cytoplasm of the trophospongia is much less intense (Plate 1, fig. E). The occurrence of the enzyme in the uterine epithelium and in the deeper coiled portions of the uterine glands is similar in amount and distribution to that described for the oestrous uterus.

Turnbull blue reagents and Perl's method for ferric iron were applied to transverse sections of three implantation sites containing the following stages: an early blastocyst in a stage of development before the formation of the embryonic knob, and an early and a late embryonic plate stage. No positive reactions for iron were obtained in any of the foetal or maternal tissues.

The vascular omphaloidean placenta

In the earlier stages of development lacunae, in which maternal blood circulates, are present in the trophoblast and trophospongia. Later in development the trophoblast becomes converted into a spongy tissue in which more numerous lacunae are formed. Maternal blood circulates in these lacunae, which are continuous with the larger blood spaces present in the trophospongia (Hubrecht, 1889). Omphaloidean villi penetrate into the trophoblast and thus bring the foetal and maternal circulations into close proximity. Generally the circulations are separated from each other by two tissues only; the trophoblast which is generally only one cell thick and the foetal endothelium. Thus in *Erinaceus* the omphaloidean placenta is haemochorial in character (Morris, 1953).

The omphaloidean placenta attains its maximum development when the embryo is about 3 mm. in length. In such stages the cytoplasm of the trophoblast and inner trophospongia is rich in RNA, but the staining is not so intense as in the early implantation stages. A moderate reaction is also obtained in the endodermal cells of the yolk-sac splanchnopleur which, in some regions of this membrane, are cuboidal in form. In the outer trophospongia there is a noticeable decrease in the amount of RNA.

The distribution of glycogen remains essentially unaltered. Glycogen is present in the inner and outer trophospongia, and a moderate staining for glycogen is obtained in the remainder of the decidual tissue. Glycogen is absent from the trophoblast, mesenchyme, and yolk-sac endoderm.

The amount of lipoidal material present in the trophoblast has increased. The rounded droplets are a little larger than those described for earlier stages and they are more numerous. As in the earlier stages they are frequently massed together to form irregularly shaped clusters (Plate 2, fig. G). Even in those regions where the trophoblast is attenuated and intervenes, together with the foetal endothelium, between the maternal and foetal circulations, the same concentration of sudanophil material is found. Similar amounts are present in the inner trophospongia, whereas in the outer trophospongia and the adjacent decidua only scattered droplets are present. The endodermal epithelium of the yolk-sac is devoid of this material. The trophoblast of the avascular bilaminar omphaloidean placenta is far more compact than in this region described above, and the concentration of sudanophil material is correspondingly more intense. Whereas in the early implantation stages of pregnancy almost all of this lipoidal material was neutral fat, at this stage of pregnancy a very large proportion of the lipoidal material present in the trophoblast and the inner trophospongia immediately adjacent to it, is phospholipid.

Alkaline phosphatase is present in the nuclei and the cytoplasm of the trophoblast at this stage, but the reaction is not so intense as that obtained in late embryonic plate stages. This also applies to the more compact trophoblast in the avascular mesometrial region of the yolk-sac wall. As in earlier stages the enzyme is present in the inner trophospongia, and in the outer trophospongia only the scattered giant nuclei, which are mainly located in this tissue, give a positive reaction. A faint reaction is obtained in the cytoplasm of the endodermal cells of the yolk-sac splanchnopleur.

No positive reactions for iron were obtained in the foetal or the maternal tissues at this stage of pregnancy. In slightly later stages of pregnancy (crown-rump length 5 mm.) a faint reaction is obtained in the trophoblast and in parts of the inner trophospongia after the application of Perl's method for ferric iron.

The yolk-sac splanchnopleur

When the vascular yolk-sac placenta attains its maximum development some of the endodermal epithelial cells of the yolk-sac splanchnopleur are cuboidal in form. At such stages the cytoplasm of these cells gives a moderate reaction after the application of methyl-green and pyronin reagents, and a similar reaction is obtained in the latest stages of pregnancy examined (crown-rump length 51 mm.). In these late stages the RNA appears to be concentrated in the distal cytoplasm of these cells, which are cuboidal or low columnar in shape. These endodermal cells rest upon a distinct basement membrane which appears to be composed of argyrophilic fibres (Plate 2, fig. J). The coelomic mesothelium of the yolk-sac splanchnopleur, which lines the membrane on its exocoelomic side is also impregnated by Gomori's silver method (1937). The coelomic mesothelium and the endothelia of the vitelline vessels are stained a clear green colour by Masson's trichrome stain and violet or red by Lillie's allochrome method for connective tissues (1951). The mesenchyme, which in the earlier stages of pregnancy was of a loose texture, is now far more compact and contains scattered argyrophilic fibres.

Glycogen is absent from the yolk-sac splanchnopleur throughout early development. However, in late stages of pregnancy glycogen is present in the endodermal epithelium and in the mesenchyme of the membrane. In both tissues it is present in the form of finely particulate material, usually occurring in the distal cytoplasm of the endodermal cells. In preparations stained with PAS reagents the basement membrane of the endodermal epithelium and the coelomic mesothelium show some variability in intensity of staining in different regions of the yolk-sac splanchnopleur. Generally both membranes are stained faintly pink by the PAS reagents, and this reaction is saliva-resistant.

The yolk-sac epithelium of the early implantation stages of pregnancy appeared to be devoid of lipoidal material, and this condition persists up to the stage at which the vascular omphaloidean placenta begins to retrogress. Subsequently lipoidal material stainable with Sudan black and Fettrot can be detected in the cytoplasm of these cells. The amount of sudanophil material present varies considerably from cell to cell, and its distribution within the cells is also variable. In some cells only a faint dusting of the cytoplasm results following the application of Sudan black, whereas in other cells large discreet droplets can be seen.

A similar result is obtained in Champy fixed material, and in Champy fixed material which was post-osmificated (Plate 2, fig. I). Generally the larger droplets are found in the lower halves of the endodermal cells, and the finer droplets are scattered throughout the distal cytoplasm. Some lipoidal material is also present in the mesenchyme. Acid haematin positive material is present in the endodermal epithelium of the yolk-sac splanchnopleur of these late stages of pregnancy. It varies in amount from cell to cell, and under high magnification this material is seen to be granular. It occurs throughout the cytoplasm of the cells, but is more concentrated in the distal cytoplasm. The amount and distribution of this granular material corresponds to that of the strongly fuchsinophil material revealed in these cells by Kull's method for mitochondria.

At the 2–3 mm. stage a faint reaction for alkaline phosphatase is obtained in the endodermal cells of the yolk-sac splanchnopleur. In later stages there is no increase in the intensity of this reaction, and the mesenchyme of the membrane appears to contain little of the enzyme. No positive reactions for iron were obtained in the membrane.

The omphaloidean placenta of late pregnancy

The rapid enlargement of the embryo in later stages of pregnancy leads to the withdrawal of the vascular omphaloidean villi, together with their surrounding mesenchyme, from their sites in the spongy trophoblast. Concurrently the extension of the exocoel separates the yolk-sac splanchnopleur from the chorion over most of its extent, and the tissues of the uterine wall become stretched—particularly in the mesometrial region.

In such late stages the trophoblast and inner trophospongia of the mesometrial region of the conceptus are indistinguishable from one another, and form a compact narrow band of tissue (Morris, 1953). Reichert's membrane has been formed on the inner embryonic side of the trophoblast.

In late stages of pregnancy the cytoplasm of the trophoblast and inner trophospongia of the mesometrial region contains little RNA. Material treated with PAS reagents reveals that glycogen is present in this tissue (Plate 3, fig. M). Large blood spaces are present between the inner and outer trophospongia and a strong PAS positive reaction is obtained in the tissue of the outer trophospongia which, in these late stages of pregnancy, is fibrous in character.

Alkaline phosphatase is present in the mesometrial trophoblast and trophospongia but in greatly reduced amounts, and the sudanophil and acid haematin positive material which bulked so large in these tissues in earlier stages is now completely lacking. Negative reactions for iron were obtained in these tissues in all the late stages of pregnancy that were tested.

Reichert's membrane

In the later stages of pregnancy in the hedgehog Reichert's membrane is formed between the somatic mesoderm and the trophoblast of the trilaminar omphalopleur. At first it is a very thin layer, but in very late stages it thickens and persists to term (Morris, 1953).

The PAS reagents stain Reichert's membrane from the time of its formation until term. At first, when the membrane is about 5 μ thick, it is stained a crimson colour, but in very late stages when it has attained a thickness of 25 μ along the lateral walls of the conceptus it is stained faintly pink (Plate 3, fig. M). A more intense reaction is obtained in the mesometrial region of the conceptus where the membrane remains relatively thin. In this region the membrane is stained a bright red colour (Plate 3, fig. N). These reactions are saliva resistant.

Gomori's silver method for the impregnation of reticulum (Gomori, 1937) impregnates the membrane in all the stages of pregnancy to which the technique was applied, though not as intensely as in the case of typical reticulum. In the lateral walls of the conceptus in late pregnancy, where the membrane is thickest, it is stained a pale brown colour with a narrow black region along its outer margin (Plate 3, fig. L), and it is stained black in the mesometrial region of the yolk-sac wall where the membrane remains relatively thin (Plate 3, fig. K).

In all stages of pregnancy Reichert's membrane is unstained by Weigert's resorcin fuchsin—the colour of the elastic tissue of the uterine vessels was used as a standard for comparison. It is stained readily by Van Gieson's stain for collagen. The membrane is clearly revealed by Lillie's allochrome method for connective tissue (1951). At the time of its first formation it is stained intensely red or violet, but in the latest stages examined the membrane is stained a pale blue colour.

In all stages of pregnancy the membrane is stained a clear green colour with Masson's trichrome stain (Plate 3, fig. O), and in preparations stained with eosin and methylene blue the membrane shows a selectivity for the acid dye. Its strong affinity for acid dyes is exhibited also in its deep staining with orange G.

In frozen sections treated with Sudan black, Reichert's membrane is completely unstained. Similarly, in material fixed in Champy's fluid and post-osmificated for varying periods, Reichert's membrane is unaffected (Plate 2, fig. H).

At the time of its formation Reichert's membrane exhibits only faint birefringence in deparaffinized sections cut from blocks fixed in Bouin's, Rossman's, and Zenker's fixatives. In unstained frozen sections cut from blocks fixed in 10 per cent. formalin and mounted moist in water or glycerogel the membrane exhibited strong birefringence, comparable with that of the reticular and collagenous fibres in the uterine wall, from the time of its formation to term. In deparaffinized sections from blocks which had been variously fixed the membrane is more strongly birefringent in very late stages of pregnancy than in earlier stages.

DISCUSSION

The occurrence of lipoids, glycogen, iron, and phosphatases in the lumina and epithelial cells of the uterine glands of a variety of mammals suggests that these substances are secreted by the glands for the purpose of nourishing the growing blastocyst during some part of the period of gestation (Wislocki & Dempsey, 1945). The 'uterine milk' of the ungulates has long been regarded as a nutritive fluid which contains lipoid, protein substances, and iron compounds. Wislocki has shown that in the sow iron and lipoid are present in the uterine glands and uterine epithelium suggesting that these substances are produced by the uterine glands and uterine epithelium, instead of being derived from erythrocytes and leucocytes which have undergone dissolution.

In *Erinaceus* the RNA content of the cytoplasm of the epithelial and sub-epithelial tissues of the uterus of hibernation is low, and there is only a slight increase in the intensity of staining in these tissues in the oestrus uterus and in the early implantation uterus. In the rat and guinea-pig an intensification of the basophilic staining of the cytoplasm of the glandular and surface epithelia of the uterus occurs prior to implantation and in many forms the subsequent conversion of the superficial stroma into decidua is accompanied by a reduction in the amount of basophilia, and the cytoplasm of the decidual cells of women, cats, rats, and guinea-pigs exhibit negligible staining with basic dyes (Wislocki & Dempsey, 1945). In the hedgehog the transformation of the sub-epithelial tissues into trophospongia is accompanied by a marked increase in the amount of cytoplasmic RNA, and this trophospongia tissue, which appears to be derived mainly from the enlarging cells of the maternal endothelium, stains as intensely as the trophoblast. The RNA content of the remainder of the decidual tissue is low.

In the hedgehog glycogen is entirely absent from the endometrium of the non-pregnant uterus, but is present in great amounts in the decidual tissues of the earliest implantation stages examined. Similarly, no positive reactions for iron were obtained in the endometria of non-pregnant uteri, and the application of Sudan black to non-pregnant uteri resulted in only a faint dusting of the distal and basal cytoplasm of the glandular epithelia. Some alkaline phosphatase is present in the surface epithelium of the uterus of hibernation and a considerable increase in the amount of this enzyme is evident in the surface and glandular epithelia, and in the lumina of the glands, of the oestrous uterus.

It is probable that the secretions of the uterine glands in all mammals contribute in some way to the nourishment of the early blastocyst, and in some degree to the support of the implanting blastocyst (Wislocki & Dempsey, 1945). Whereas in some animals such as the sow and other centrally implanting forms, the uterine glands may serve as a principal source of nutritive materials, in others they play only a subsidiary role. In *Erinaceus*, in which implantation is of the interstitial type, it appears that the uterine secretions are of minor importance since it seems unlikely that they are rich in nutrients. The secretions of the glands are available to the blastocyst for only a short time, since the early establishment of the decidua capsularis effectively separates the foetal tissues from the uterine lumen and its fluid contents. Once implantation commences other means of obtaining nutritive materials become available.

In the early stages of pregnancy in *Erinaceus* the yolk-sac, which is a closed cavity, is dependent for any renewal of its fluid contents as far as maternal sources are concerned, on substances which enter it from the maternal blood, passing through the trophoblast and endoderm. Since numerous lacunae, in which maternal blood circulates, are present in the trophoblast and trophospongia of such early stages, the early omphaloidean placenta should be an important source for the transudation of fluid into the yolk-sac cavity. Everett (1935) contends that that proximity of circulating maternal blood to Reichert's membrane in the early stages of pregnancy in the rat accounts for the rapid appearance of vital dyes in the cells of the yolk-sac endoderm and in Reichert's membrane itself.

In all the implantation stages examined the trophoblast, trophospongia, and the enlarging cells of the maternal endothelia are rich in RNA. RNA is important in the process of protein synthesis (Danielli, 1953), and in these tissues it is probable that it plays some important role in the synthesis of protein during the early period of active growth of the blastocyst and decidua.

The trophoblast and trophospongia are rich in neutral fat although this material is absent from the yolk-sac endoderm. The individual droplets are frequently clustered together into large irregularly shaped masses, and no specific polarity is indicated by the distribution of this material within the cells. The amount of lipoidal material present in individual cells varies considerably. It is probable that these droplets are absorption products, and that the early omphaloidean placenta is concerned in the absorption of lipoidal material from the maternal blood. The vascular omphaloidean placenta is also important in this connexion, for in later stages of pregnancy (crown-rump length 2–5 mm.) the trophoblast and trophospongia contain increased amounts of lipoidal material, a large proportion of which is phospholipid.

The role of alkaline phosphatase in tissue metabolism is as yet inadequately known. It is thought that phosphatases are probably involved in a variety of chemical reactions which occur in tissue metabolism and the specificity of their actions cannot be demonstrated by histochemical means. It is not therefore possible to attribute a specific function to the tissues in which the enzymes are found. Probably phosphatases play some part in the conversion of glycogen into glucose (Cori, 1941), and there is some evidence to show that sugars are phosphorylated in their absorption into the epithelia of the intestine and kidney tubule (Davson & Danielli, 1943). It has been suggested that fats undergo a like phosphorylation (Bloor, 1943), and that the phosphate necessary in this scheme of sugar and fat absorption is presumably made available by phosphatases (Sumner & Somers, 1943). In view of these activities the sites of enzyme location which merit attention as far as the yolk-sac placenta is concerned, are the trophoblast and trophospongia.

The concentration of alkaline phosphatase in the trophoblast increases as pregnancy proceeds, and the enzyme is present in this tissue in considerable amount at the late embryonic plate stage. A somewhat less intense reaction is obtained in the trophoblast at the stage in which the yolk-sac placenta attains its greatest development. The enzyme is also present, in lesser amounts, in the trophospongia. The alkaline phosphatase in these tissues may play some part in the absorption of sugars from the maternal blood in the early avascular yolk-sac placenta and in the vascular yolk-sac placenta which is developed later. In late stages of pregnancy the trophoblast and trophospongia of the yolk-sac wall contain very little of the enzyme, and this reversal of concentration suggests in itself a significant change in the physiology of the yolk-sac wall during the course of gestation.

In *Erinaceus* the trophospongia is very rich in glycogen and the amount of this substance present—judging by the intensity of the staining reaction with PAS reagents and Best's carmine—appears to increase between early implantation and late embryonic plate stages. The source of glucose for the elaboration of the glycogen molecule must be the maternal blood. It is probable that a similar uptake of sugars occurs in the trophoblast in which greater amounts of alkaline phosphatase are present, and that it is utilized by this tissue, for glycogen deposition does not occur in it until very late in pregnancy when the yolk-sac placenta is in an advanced state of retrogression.

It is generally considered that decidual tissue, which is invariably rich in glycogen and fat, plays an important part in the nourishment of the early blastocyst. It has been suggested (Hard, 1946) that the presence of alkaline phosphatase in this tissue may indicate the existence of a mechanism whereby these substances may be released and made available to the blastocyst. The relatively reduced amount of glycogen in the trophospongia of the vascular yolk-sac placenta—when compared with that of the avascular placenta of embryonic plate stages—may be correlated with this.

In *Erinaceus* the yolk-sac persists to term and in late stages the yolk-sac splanchnopleur is invaginated into the yolk-sac and becomes thrown into folds. At term the yolk-sac appears in transverse sections as a slit-like crescentic cavity, the bilaminar (mesometrial) wall of which is complete (Morris, 1953). The yolk-sac placenta of the hedgehog is concerned in the absorption of lipoidal material from the maternal blood, yet lipoidal droplets have not been detected in the endoderm of the yolk-sac until very late in pregnancy. It therefore appears unlikely that the lipoids absorbed by the trophoblast are transmitted into the yolk-sac cavity, and it is probable that the lipoidal material that accumulates in the endoderm and mesenchyme of the yolk-sac splanchnopleur in late pregnancy is derived from the foetal blood of the vitelline circulation. A similar consideration may be applicable to the late occurrence of glycogen in the membrane.

The yolk-sac splanchnopleur does not become fully differentiated until late in development when the vascularity of the mesometrial half of the conceptus is poor and the vascular yolk-sac placenta has retrogressed. Very little alkaline phosphatase is present in the endoderm of the membrane at the 2-mm. stage, and there is no apparent increase in the amount of the enzyme in the endoderm in later stages. Inorganic iron is absent from the membrane in all the stages that were tested.

It is unlikely that the yolk-sac fluid in late stages of pregnancy can be regarded as an important source of absorbable substances. It has been shown (Morris, 1953) that the concentration of protein nitrogen in the yolk-sac fluid increases in late pregnancy, and that this increase is probably due in part to the absorption of water from the cavity. Since this cannot account for the higher concentrations obtained, there must be a continued entry of protein into the yolk-sac in late stages. The probable route of entry is from the vitelline vessels of the yolk-sac splanchnopleur.

The endodermal epithelial cells of the yolk-sac splanchnopleur of rodents contain glycogen, fat droplets, and iron, and it has been shown in the rat and the hamster (Wislocki *et al.*, 1946) that this epithelial layer is probably concerned in the absorption of iron. In those forms in which the yolk-sac becomes inverted, the vascular yolk-sac splanchnopleur is either bathed by the fluid contents of the uterine lumen, which may contain a variety of substances capable of being absorbed, or it may come to lie adjacent to the decidua capsularis. It has been suggested (Wislocki *et al.*, 1946) that the yolk-sac splanchnopleur of these forms is actively engaged in the absorption of iron, fat, and protein from the uterine fluid or from the yolk-sac cavity, the bilaminar wall of which has degenerated. In the hedgehog it is probable that the yolk-sac splanchnopleur is not of any great importance as a placental structure.

In *Erinaceus*, as in some of the shrews (Brambell & Perry, 1945), Reichert's membrane is formed very much later in development than in the various rodents in which it occurs, and it first becomes noticeable between the somatic mesoderm and the trophoblast of the lateral wall of the conceptus (Morris, 1953). In so far as the yolk-sac serves as an absorptive organ the conditions under which substances may reach it will differ materially before and after the appearance of Reichert's membrane. The yolk-sac placenta is of very great importance in the nourishment of the growing blastocyst in early stages of pregnancy, and it continues to be of importance up to about the 7-mm. stage. Subsequently its importance rapidly declines as the extent of the vascular omphaloidean placenta diminishes. In *Erinaceus* the formation of Reichert's membrane is more or less coincident with the onset of the retrogression of the omphaloidean placenta, and in this species the membrane attains a far greater thickness than in the rat. In later stages of pregnancy the vascular flow in the omphaloidean (mesometrial) region of the conceptus is being retarded. These factors strongly suggest that the entry of substances via the omphaloidean placenta is very seriously curtailed from the stage of pregnancy in which Reichert's membrane appears onwards.

The histochemical properties and tinctorial reactions of Reichert's membrane have been described. The staining reactions of the membrane are, in some ways, similar to those described for Reichert's membrane in the rat (Wislocki & Padykula, 1953). The failure of the membrane to stain with Weigert's resorcin fuchsin indicates that it is not composed of elastic tissue. Its marked affinity for acid dyes, its reaction to Van Gieson's stain, its argyrophilia, and its birefringence suggest that it is composed of collagenous material. In the rat the membrane does not stain typically as dense collagen or as reticulum (Wislocki & Padykula, 1953), whereas in *Erinaceus* the staining reactions of the membrane are far more typical and very similar to those of collagen.

The membrane is strongly PAS positive at the time of its formation, indicating that it probably contains some mucopolysaccharide or glycoprotein. This strong reaction persists to term in those regions of the mesometrial wall of the conceptus where the membrane remains comparatively thin (5–15 μ). However, along the lateral walls of the conceptus where it may reach a thickness of 30 μ, it is stained faintly pink with PAS reagents after saliva treatment. It has been assumed that reticular fibres are strongly PAS positive, whereas collagenous bundles are poorly stained. It is probable that the degree of PAS staining of collagenous tissues is dependent upon the presence of ground substance coating the connective tissue fibres as well as upon the fibres themselves (Lillie, 1952).

Silver impregnation techniques have been used in attempts to distinguish between reticulum and collagen in connective tissues. The reticular fibres are blackened by silver impregnation, whereas collagenous bundles are stained brown—the colour may vary from light golden brown to dark brown. In the later stages of pregnancy in *Erinaceus* to which Gomori's silver impregnation method was applied, Reichert's membrane is stained black in those mesometrial regions of the conceptus where the membrane is relatively thin, and a pale brown colour along the lateral walls of the conceptus where it is very much thicker. It has been shown (Irving & Tomlin, 1954) that the difference in the silver impregnation of reticulum and collagen is not a means of differentiating between two types of fibres, but only distinguishes between two types of fibre ground-substance complex, and that these different responses of reticulum and collagen cannot be held to contradict the conclusion that the fibres of reticulum and collagen are identical. Thus it seems probable that the presence of varying amounts of ground-substance complex—relative to the thickness of the membrane—may account for the different reactions of the membrane to PAS reagents and to silver impregnation in the mesometrial and lateral regions of the wall of the conceptus.

In the rat (Wislocki & Padykula, 1953) the membrane exhibits variable birefringence, which is not as constant or as strong as that of reticular and collagenous fibres. In *Erinaceus* the membrane exhibits birefringence at the time of its formation and it becomes more strongly birefringent in late stages of pregnancy. In such late stages its birefringence is comparable to that displayed by the reticular and collagenous fibres present in the uterine wall.

The origin of Reichert's membrane in the rat and mouse is uncertain. It may arise from the endoderm of the bilaminar omphalopleur or from the trophoblast. In some of the shrews (Brambell & Perry, 1945) and in *Erinaceus* (Morris, 1953), there is strong evidence that the membrane is of trophoblastic origin.

SUMMARY

1. An account is given of the distribution of RNA, fats, iron, glycogen, and alkaline phosphatase in the endometria of non-pregnant animals and in the foetal tissues and decidua of the yolk-sac placentae of pregnant forms.

2. The RNA content of the cytoplasm of the uterine and glandular epithelia of the oestrous uterus of *Erinaceus* is low, and little lipoidal material is present. Iron and glycogen are entirely absent. The uterine secretions must play only a minor role in the nourishment of the early blastocyst.

3. The histochemical reactions of the tissues which comprise the avascular yolk-sac and the vascular yolk-sac placentae are described. The yolk-sac placenta is concerned in the nourishment of the embryo in early stages of pregnancy —up to about the 7-mm. stage, for it is involved in the uptake of substances, particularly fats and sugars from the maternal blood. Subsequently, the yolk-sac placenta retrogresses. Whereas in early stages the trophoblast of the yolk-sac wall is rich in alkaline phosphatase in late stages very little of the enzyme is present in this tissue.

4. In *Erinaceus* the yolk-sac fluid of late stages of pregnancy cannot be regarded as an important source of absorbable substances, and it is improbable that the yolk-sac splanchnopleur, the histochemical reactions of which are described, is as important as a placental structure in this species as it is in the rodents.

5. The histochemical and tinctorial reactions of Reichert's membrane are described, and these reactions are compared with those that have been described for the membrane of Reichert formed in the rat. Since the membrane is unstained by Weigert's resorcin fuchsin elastic tissue is not one of its components. Most of its observed properties suggest that it is formed of collagenous fibres. In the hedgehog its formation is more or less coincident with the onset of the retrogression of the yolk-sac placenta.

I wish to express my thanks to Professor F. W. R. Brambell, F.R.S., for his advice.

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EXPLANATION OF PLATES

PLATE 1

Abbreviations:

E.	Endoderm	*MS.*	Maternal blood space	*Tr.*	Trophospongia
M.	Maternal blood	*R.*	Reichert's membrane	*V.*	Vitelline vessel
Me.	Mesoderm	*T.*	Trophoblast	*Y.*	Yolk-sac cavity

FIG. A. Part of a transverse section of the uterus of a hibernating female showing the presence of alkaline phosphatase in the uterine epithelium and in the necks of the uterine glands. Cold acetone fixation. ×90.

FIG. B. Part of a transverse section of an oestrous uterus showing the distribution of the greatly increased amount of alkaline phosphatase. The enzyme is now present in the deeper parts of the uterine glands. Cold acetone fixation. ×80.

FIG. C. Part of the bilaminar wall of a very early blastocyst. Very little alkaline phosphatase is present in the endoderm, trophoblast, and trophospongia. Formalin fixation. ×450.

FIG. D. Part of the bilaminar wall of a later blastocyst showing the increased amount of alkaline phosphatase in the trophoblast and trophospongia. Formalin fixation. ×340.

FIG. E. Part of the bilaminar wall of a late embryonic plate stage in which the endoderm and trophoblast are particularly rich in alkaline phosphatase. Cold acetone fixation. ×320.

PLATE 2

FIG. F. Part of the bilaminar wall of an early blastocyst showing the distribution of lipoidal material in the trophoblast and trophospongia. Formalin fixation and stained with Sudan black. × 525.

FIG. G. Part of the vascular yolk-sac placenta at its greatest development showing the increased amount of lipoidal material present. Formalin fixation and stained with Sudan black. × 315.

FIG. H. Part of the lateral wall of a late conceptus showing Reichert's membrane and the absence of lipoidal material in the trophoblast and trophospongia. Champy fixation and post-osmification. × 370.

FIG. I. Part of the yolk-sac splanchnopleur of a late stage of pregnancy showing the lipoidal material present in the endoderm and mesenchyme. Champy fixation and post-osmification. × 500.

FIG. J. Part of the yolk-sac splanchnopleur of a late stage showing the argyrophilia of the basement membrane of the endoderm and of the coelomic mesothelium. Argyrophilic fibres are present in the mesenchyme. Formalin fixation and silver impregnation. × 400.

PLATE 3

FIG. K. Part of the mesometrial wall of the yolk-sac of a late stage of pregnancy. Reichert's membrane is coloured black after silver impregnation. Formalin fixation. × 450.

FIG. L. Part of the lateral wall of a late conceptus in which Reichert's membrane is coloured brown after silver impregnation. Formalin fixation. × 470.

FIG. M. Part of the lateral wall of a late conceptus. Reichert's membrane is stained faintly pink and glycogen is present in the trophoblast and trophospongia. Rossman fixation and PAS reagents. × 440.

FIG. N. Part of the mesometrial wall of a late conceptus. Reichert's membrane is stained crimson. Rossman fixation and PAS reagents after treatment with saliva. × 440.

FIG. O. Part of the yolk-sac wall showing Reichert's membrane, which is stained green, soon after it first becomes discernable. Bouin fixation and stained with Masson's trichrome. × 360.

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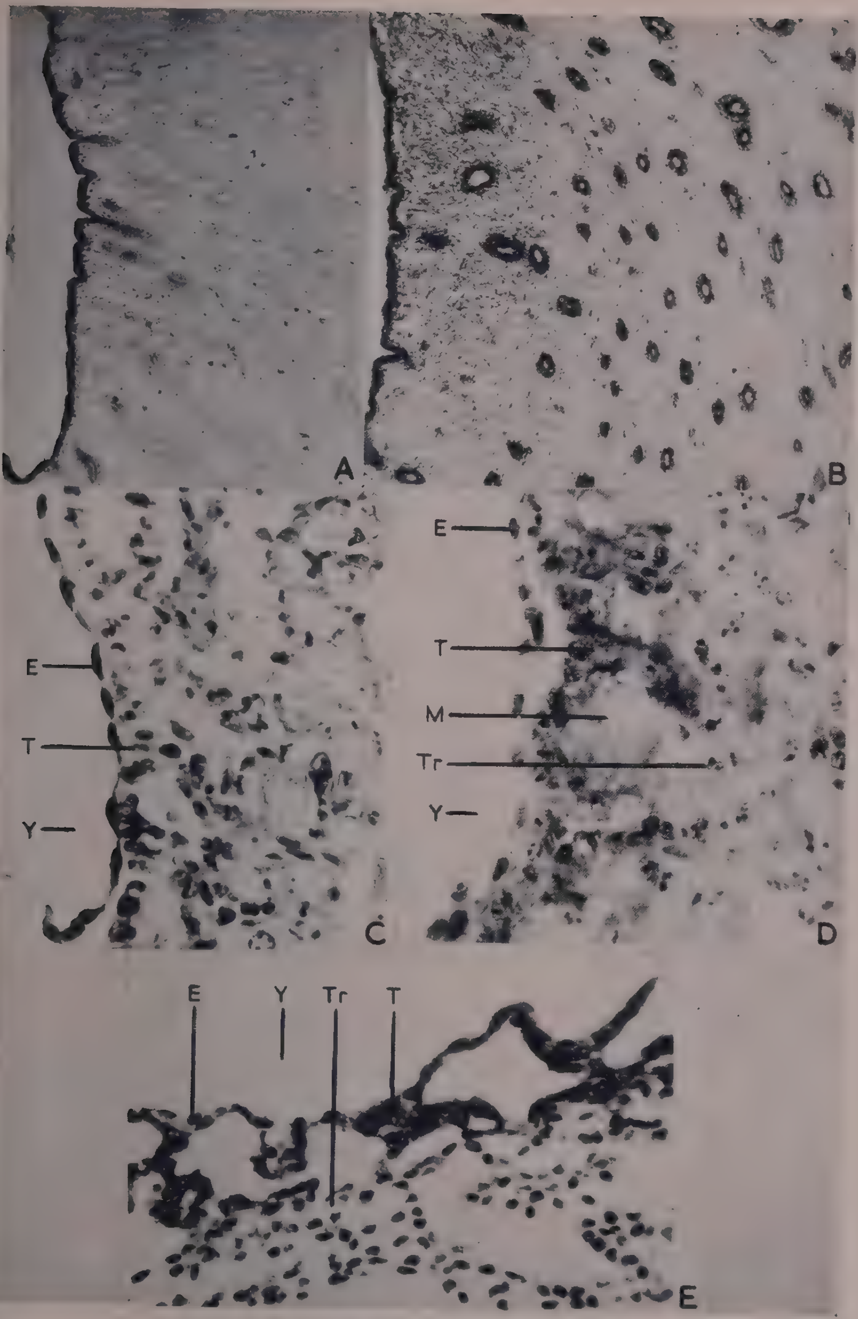
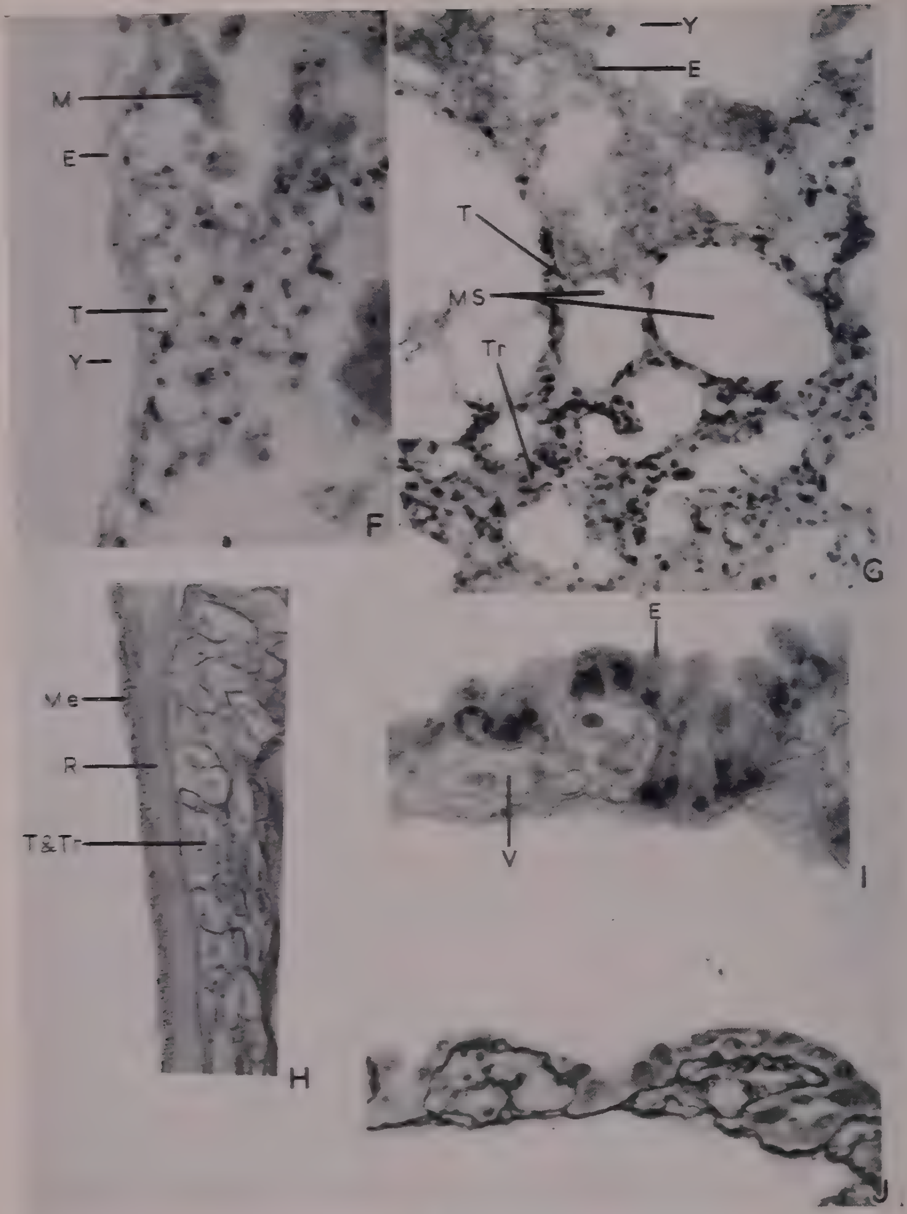


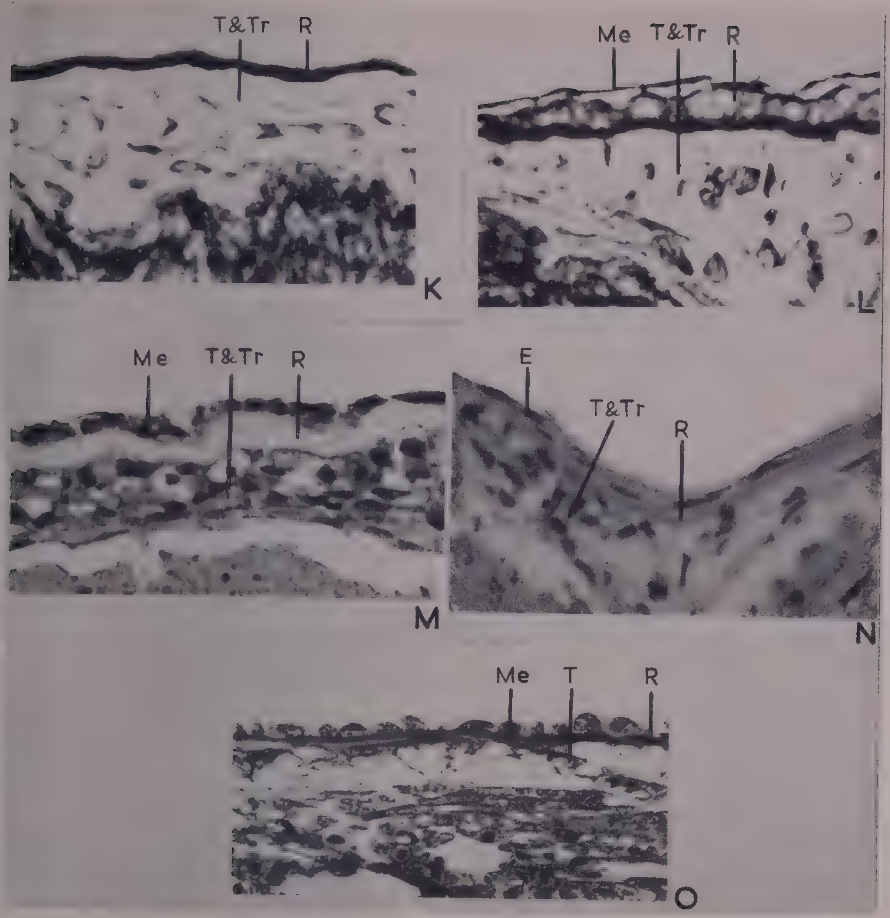
B. MORRIS

Plate 1



B. MORRIS

Plate 2



B. MORRIS

Plate 3

The Embryology of Cortisone-induced Cleft Palate

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WITH TWO PLATES

INTRODUCTION

In the past, the sporadic, unpredictable occurrence of cleft palate made the study of its embryological origins difficult. The discovery by Fraser *et al.* (1954) that large doses of cortisone injected into pregnant strain A/Jax females would cause cleft palate in 100 per cent. of the offspring, provided a means for studying cleft palate in all stages of its development. Also, the lower incidence (17 per cent.) of this defect in strain C57BL after cortisone treatment supplied an additional tool for detecting differences between normal and abnormal palate development. Thus, a study of the embryology of cortisone-induced cleft palate was undertaken.

However, to understand the abnormal it is usually necessary first to understand the normal. Since the literature on normal palate development was incomplete and controversial, this subject was re-examined and the results have been presented previously (Walker & Fraser, 1956). In summary, this study showed that the palatine shelves, which initially lie in a vertical plane lateral to the tongue, move into a horizontal plane extending across the tongue dorsally, where they meet in the midline and fuse. This movement is accomplished by a protrusion of the dorso-medial wall of each palatine shelf and a compensating regression of the ventral ridge, with the movement of shelf tissue and depression of the tongue being accomplished by some force within the shelves. It is recommended that the reader refer to Walker & Fraser (1956) before reading the following account of cleft palate.

MATERIALS AND METHODS

The mice used in this experiment were of strains A/Jax and C57BL/Fr. Pregnant female mice were injected intramuscularly with daily doses of 2·5 mg. of cortisone acetate (Merck) at 10 a.m. on days 11 to 14 of gestation, inclusive. Other procedures were the same as in our previous paper.

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RESULTS

1. *Effect of treatment on shelf movement*

Comparisons were made between embryos of treated and untreated mothers, 15–17 days post-conception. Embryonic age was calculated on the assumption that conception occurred at 2 a.m. on the morning a vaginal plug was seen (Snell *et al*. 1940). The day on which a vaginal plug was observed was counted as day 0. Plate 1, fig. A shows an untreated embryo during the process of palate closure on day 15, 4 hours post-conception. One shelf has reached the horizontal plane, but the other is still mainly in the vertical plane. (This is called stage 3 of palate closure by the criteria listed in our previous paper.) Plate 1, fig. B shows a treated embryo of the same strain observed at the age of 16 days 8 hours post-conception, in which the shelves have not even begun to move toward the horizontal though in an untreated animal the palate would have long since closed. That this is not an error in timing is clear from the fact that the head is much larger and the shelves bulkier in the treated than the untreated specimen, indicating that the treated embryo is older, even though its palate has not yet begun to close. The shelves eventually become horizontal (Plate 1, fig. C), but do not touch and fuse. (Apparently cortisone delays shelf movement, but growth of the head continues so that when the shelves do reach the horizontal they are too far apart to meet in the midline.) The larger, older embryo heads show a progressively increasing distance between the medial edges of the shelves (Plate 1, fig. D).

The delay in palate closure described above can also be seen in histological preparations. The section shown in Plate 2, fig. E was taken from a cortisone-treated embryo in which palate closure had already been delayed about one day. One shelf is horizontal, but the other shelf is still in transition from the horizontal to the vertical plane, and the protrusion of a medial bulge with the compensating regression of the ventral portion (as described previously by Walker & Fraser, 1956) can be observed. Completion of shelf movement would leave the shelves much too far apart to fuse.

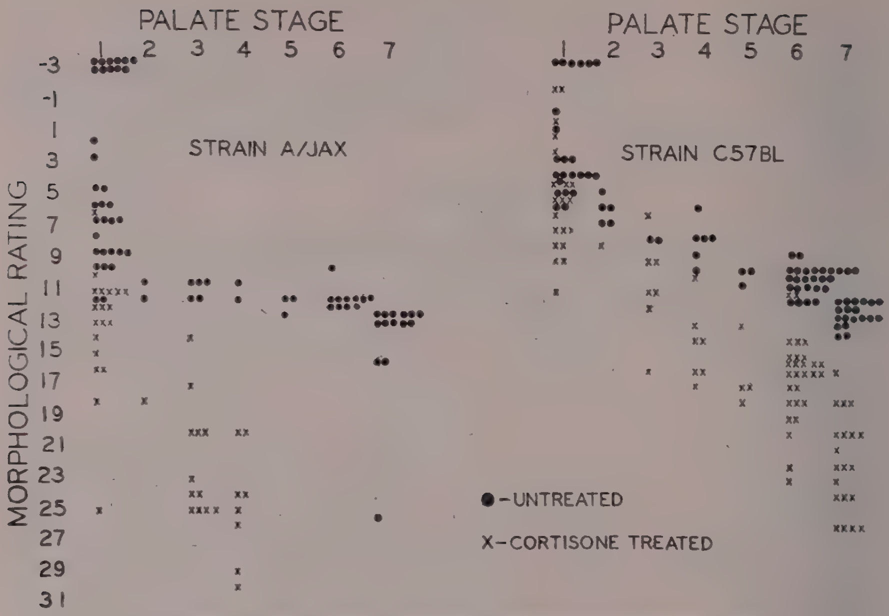
The disturbance in development suggested by the above individual observations becomes clearer when the relation of palate stage to chronological age is plotted for a number of embryos. Table 1 presents the relationship of stage of palate closure to chronological age in 40 strain A/Jax embryos and 87 strain C57BL embryos of mothers treated with cortisone. In untreated strain A/Jax animals, the oldest embryos observed that had not begun to close were 15 days 8 hours old, and some were already closed by 14 days 20 hours (Walker & Fraser, 1956). However, it can be seen from Table 1 that the treated A/Jax embryos never get past stage 4 (both shelves horizontal but not touching), and some have not begun to close at 16 days and 8 hours. Thus it is clear that the cortisone delays initiation of shelf movement. However, it also seems to slow down the process of closure, as indicated by the higher frequency of stage 3 in treated A/Jax embryos (12 in 40) than in the controls (5 in 58). This is an intermediate, short-lasting stage where one shelf has already moved to the horizontal plane while the other is still vertical. The higher frequency of stage 3 in the treated animals suggests that they take longer to go through this stage. In strain C57BL a similar but less extreme slowing down of the process of closure is evident (Table 1).

TABLE 1

Relation of palate stage to chronological age

Days/hours post-conception	*Cortisone-treated A/Jax embryos*							*Cortisone-treated C57BL embryos*						
	Palate stage							*Palate stage*						
	1	2	3	4	5	6	7	1	2	3	4	5	6	7
13/8														
13/16														
14/8								4						
14/10								2				1	4	1
14/12														
14/14								2	1			1		3
14/16								2			1			2
14/18								3		2	1		1	
14/20	4							6		3	1		2	1
14/22											1		5	
15/0														
15/2								1					4	
15/4	6	1	1											
15/6	5									1	2	1	2	1
15/8			2	1								1	8	5
15/10														
15/12														
15/14										1	1		1	3
15/16	2		2	1										
16/8	2		7	4									1	4
17/8				2										

Ratings by morphological criteria of developmental stage provide a more accurate scale than chronological age for following the progress of normal palate closure (Walker & Fraser, 1956) and this can be seen to apply also for cortisone-treated embryos. In Text-fig. 1, when the distribution of cortisone-treated embryos is compared to the distribution of normal embryos, it is clear that cortisone has caused marked delay in the progress of palate-closing relative to other criteria of embryonic development. Since the palate had started fusing by morphological rating 13 in all normal embryos examined, it might be suspected that those treated embryos which are beyond morphological rating 13 but still not up to stage 5 (shelves touching) are the embryos that would have been born with cleft palate. Since no treated A/Jax embryos reach stage 5, they should all be born with cleft palate (which, in fact, they are), whereas the relatively few treated C57BL embryos observed with retardation of shelf movement beyond this critical period presumably represent the relatively few C57BL offspring observed with cleft palate at birth.



TEXT-FIG. 1. Scatter diagram showing the relationship between palate stage and morphological rating in treated and untreated embryos from two strains of mice.

The palate stage numbers indicate degree of palate closure from a completely open palate at stage 1 to a closed and fused palate at stage 7. Morphological ratings consist of the sum of numbers given to the various external features of the embryo such that the higher the number the more advanced is the embryo developmentally (for details see Walker & Fraser, 1956).

The head and palatine shelves were measured to see if cortisone influenced their growth rate. There was no evidence for growth retardation in the shelves of cortisone-treated embryos up to the time of normal palate closure. Beyond this point there was relative reduction in shelf width in those embryos where shelf fusion did not take place. That is, the shelves seem to grow very little in width unless they first fuse.

When a living embryo is dissected out of the uterus shortly before the normal time of palate closure and its tongue is removed from between the palatine shelves, the palatine shelves will move from a vertical to a horizontal position. When this experiment was performed on a litter of cortisone-treated embryos of an estimated 14 days 21 hours post-conception (morphological rating 8), the shelves did not move, although they did so in untreated embryos of that age (Walker & Fraser, 1956). However, in cortisone-treated embryos 15 days 8 hours old (morphological rating 20) most of the palatine shelves, which would have been horizontal and fused in untreated embryos, could move to the horizontal plane when the tongue was removed.

2. *Injection of cortisone into the amnion*

It would be advantageous to know if cortisone can produce cleft palates by acting directly on the embryo or whether it must first act on the mother. Injecting cortisone into the amniotic fluid seemed to be the best way of having it reach the embryo without causing a high frequency of resorption. (A few trial injections directly into embryos caused resorption in all cases.)

The uterus was exposed through a midline abdominal incision in pregnant mice at 12–14 days post-conception, and injections of cortisone were made through the uterine wall and amnion (as estimated by visual observation and by the leaking of amniotic fluid) in one uterine horn, while injections of cortisone vehicle were similarly made in the other uterine horn. Injections were made through a size 25 hypodermic needle attached to a 1 c.c. glass syringe on which a screw-clamp was mounted. A single rotation of the screw-clamp delivered approximately 1/100 c.c. The females were killed several days after the operation and the uteri with the contained embryos were put in a fixative. Surprisingly, cleft palates appeared in embryos injected with cortisone vehicle as well as those injected with cortisone. Presumably, leaking of amniotic fluid through the hole made by the hypodermic needle reduced the hydrostatic pressure in the amnion, thus allowing the contracting uterus to press down on the embryo. This conclusion was reached because the embryos with punctured amniotic sacs appeared to have been subjected to considerable pressure, as is illustrated by the embryo in Plate. 2, fig. F. This contraction would tend to press the head down on the chest, thus making it more difficult for the shelves to displace the tongue. The results ranged from a cleft palate where the shelves were horizontal but not touching to the extreme condition seen in Plate 2, fig. G, where the tongue is greatly distorted by pressure and remains tightly wedged between the shelves. Thus we were unable to find out, by this method, whether cortisone affects the embryo directly. However, the results do suggest another mechanism for the production of cleft palate, which is being investigated further.

3. *Effect of position in uterus*

One of the puzzling things about congenital defects is that although all embryos of an inbred litter have a relatively homogeneous genotype and uterine environment, only a few of them may actually develop a defect when exposed to a genetic or environmental teratogen. This phenomenon is seen in litters from cortisone-treated C57BL females, where approximately one new-born mouse out of five has a cleft palate. Similarly, in litters from normal C57BL mice, some individuals may have microphthalmia, although this is an inbred strain. To discover whether the location of an embryo in the uterus relative to its litter mates influenced which embryos would become defective, a survey was made of the location of defective embryos in untreated C57BL and A/Jax mothers. Conditions considered to be defects were cleft lip, microphthalmia, anophthalmia, micrognathia, and embryonic resorptions down to the size where a lump in the uterus was still plainly visible to the unaided eye. Only uterine horns with three or more embryos were included in the data, so that each horn had one embryo adjacent to the vagina, one embryo adjacent to the ovary, and one or more embryos intermediate to these two positions. The only litters considered were those with at least one defective embryo. Results are given in Table 2.

TABLE 2

	Position of embryos in uterus		
	Ovarian	*Intermediate*	*Vaginal*
Normal embryos . . .	71	167	71
Defective embryos . . .	19	48	23

The total scores for defective embryos in ovarian (21 per cent.), intermediate (22 per cent.), vaginal (24 per cent.) positions do not differ significantly from one another ($P = 0{\cdot}90$). Thus no evidence has been found to indicate that the embryo's relative position in the uterus is a significant factor in the appearance of developmental defects.

DISCUSSION

When pregnant mice of strain A/Jax received a particular schedule of cortisone injections, the result was a delay in the average time and rate at which palate shelf movement occurred. A similar but not less severe situation occurred in strain C57BL. This is, no doubt, the primary cause of cleft palate in the new-born mice from cortisone-treated mothers. A delay in the movement of the shelves to meet each other in the midline, plus continuing growth of the head, may result in the shelves being so far apart when they reach their final position that they are unable to fuse. Retardation in shelf movement is most striking in A/Jax embryos, as would be expected since they develop the greatest number of cleft palates after cortisone treatment. This is evident in:

(1) the photographs of certain embryos (Plate 1, figs. B, C, D) that are obviously advanced in age for the state of their palate development;

(2) the tables of chronological age of embryos at various palate stages (cf. Table 1 and Walker & Fraser, 1956);

(3) the graph of morphological development relative to palate stage (Text-fig. 1).

Table 1 and Text-fig. 1 also show that the speed of shelf movement is greatly decreased, so that stage 3 (one shelf vertical, one horizontal), which is normally rather infrequent, is found throughout a wide range of morphological and chronological ages. As to the manner in which cortisone treatment interferes with shelf movement, there are the alternative possibilities of direct inhibition within the shelves of the force causing shelf movement (Walker & Fraser, 1956), or an increase in the resistance of the tongue to being displaced. That this latter factor can delay or prevent shelf movement was strongly suggested by experiments in which cleft palates had occurred in embryos where the pressure of amniotic fluid had been reduced, thus subjecting the embryos to increased pressure from the uterus, which forced the head and lower jaw against the chest, pressed the tongue dorsally, and therefore increased the resistance of the tongue to being displaced by the palatine shelves. However, experiments on living embryos from cortisone-treated mothers indicated a reduced potentiality for shelf movement even when the tongue was withdrawn.

If cortisone treatment were affecting the shelves directly, it might inhibit the build-up or the release of the force causing shelf movement. The palatine shelves at the time of palate closure consist of connective tissue covered with epithelium. Cortisone is known to reduce the metachomasia and fibrillogenesis in connective tissue during wound healing (Ragan *et al.*, 1950). Since the connective tissue of the palatine shelves displays metachromasia and reacts to Gomori's elastic tissue stain, it may be that cortisone inhibits the growth of fibres or the consistency of the ground substance. More information has to be assembled before conclusions can be reached on this topic, especially since the fibrous nature of the material reacting to aldehyde fuchsin staining in the palatine shelves is not clearly established. The most promising approaches are comparative studies using histochemical techniques, and experimental techniques on living embryos.

Cortisone may not have to reach the embryo directly to exert its teratogenic action. Benditt *et al.* (1950) found a minimum of 24–48 hours of pretreatment with cortisone was necessary to produce decreased capillary permeability, and suggested an intermediate step might account for the lag between administration and effect. A similar intermediate step might occur in the pregnant mouse, with the resulting products then reaching the embryonic connective tissue. However, the work of Jones *et al.* (1953) indicates that cortisone does cross the placenta and could therefore act directly on the embryonic palate.

Measurements of the palate region have not shown any sign of growth retardation due to cortisone treatment until after the time of palate closure. The retardation of shelf movement results in cleft palate because (*a*) fusion of the shelves is apparently necessary to stimulate continued shelf growth beyond the size seen during normal palate closure, and (*b*) head growth is not noticeably affected by cortisone during this period; therefore, the late moving shelves are drawn apart by increasing head width, and when the shelves finally become horizontal they are too far apart to touch and fuse.

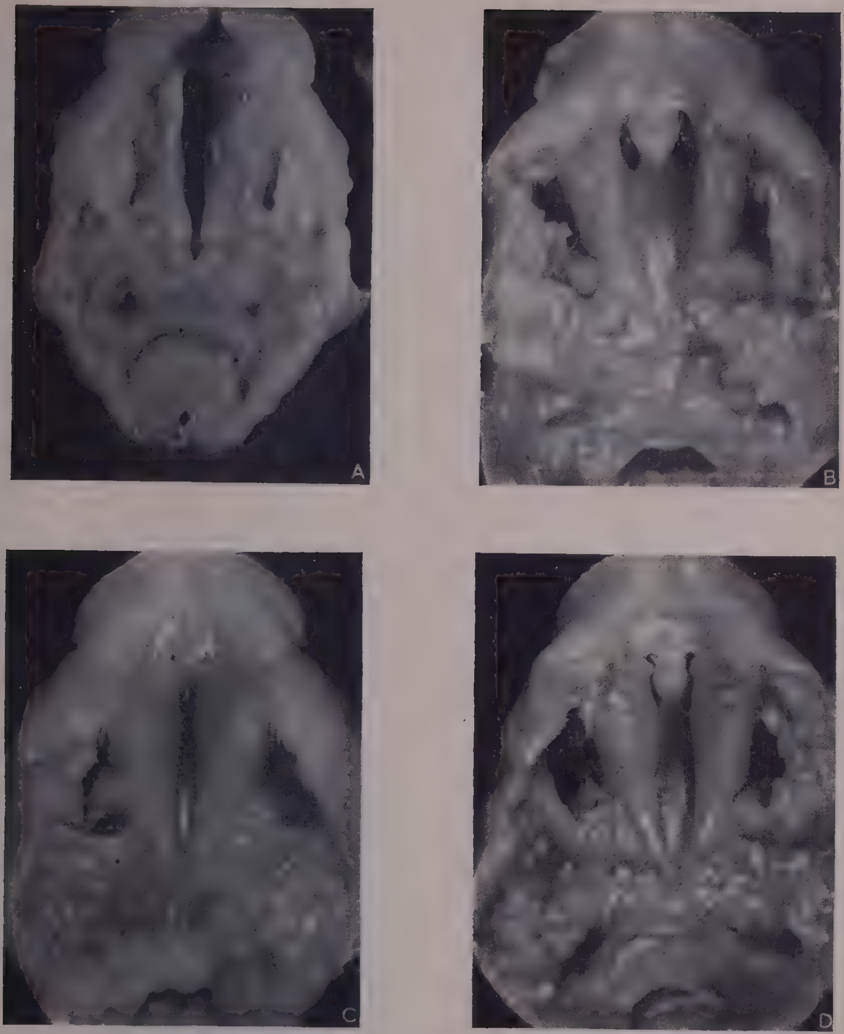
Shelf movement is sufficiently delayed in A/Jax embryos to cause a very high incidence of cleft palate. In C57BL embryos delay also occurs (Table 1 and Text-fig. 1), but apparently fusion can take place sufficiently later in development to enable most embryos to produce normal palates. If the variability in the developmental stage at which palate closure takes place as shown in Text-fig. 1 is accepted as real, it may be assumed that when cortisone delays the average time of closure, the shelves that would normally have been late in moving would then move too late to fuse at all. The earlier closing of C57BL palates may be a reflection of a greater ability of this maternal-embryo system to build up the force necessary for shelf movement as compared to the A/Jax strain. If this were true, then it could be deduced that C57BL strain has a more efficient developmental system in the shelves to counteract the inhibiting effects of cortisone. One might suggest that palate closure could be delayed in C57BL embryos up to the normal time for palate closure in A/Jax embryos without preventing shelf fusion, and that this accounts for the strain difference in incidence of cleft palate. However, the data in Table 1 and Text-fig. 1 indicate that the extra potential time for palate closure gained by C57BL embryos through early closure is not sufficient to account completely for the strain differences in the effect of cortisone on C57BL and A/Jax embryos. For example, C57BL palates normally start closing about 12 hours earlier (Walker & Fraser, 1956) than A/Jax palates, but after cortisone treatment stage 1 palates occur in A/Jax embryos as much as 30 hours (cf. Table 1) later than the latest stage 1 palate observed in C57BL embryos. Similarly, the appearance of stage 3 (one shelf vertical, one horizontal) at much more advanced ages (morphologically and chronologically) in A/Jax embryos than in C57BL embryos suggests that the palate shelf tissue in this strain is less able to resist the effects of cortisone than is the shelf tissue in strain C57BL.

SUMMARY

Cortisone treatment causes cleft palates by interfering with the development of the condition within the palatine shelves that forces them to change their alignment from the vertical to the horizontal plane. The consequent delay in shelf movement, the apparent inability of the shelves to grow wider without the stimulus of being fused, and the continued increase in head width, result in a palate in which the shelves are too far apart to touch and fuse when they finally become horizontal.

The strain difference between C57BL and A/Jax mice in their susceptibility to the teratogenic action of cortisone is due in part to the earlier closing of the palate in C57BL embryos, and in part to the lesser inhibition of shelf movement in C57BL embryos as compared to A/Jax embryos. These differences suggest that the C57BL maternal-embryo complex has a more efficient metabolic system for developing palate shelf movement than exists in the A/Jax strain.

The financial assistance of the Kate E. Taylor Fund of the Banting Research Foundation, and of the National Research Council of Canada, and of the Faculty of Medicine, McGill University (Blanche Hutchison Fund) is gratefully acknowledged.



B. E. WALKER *and* F. C. FRASER

Plate 1

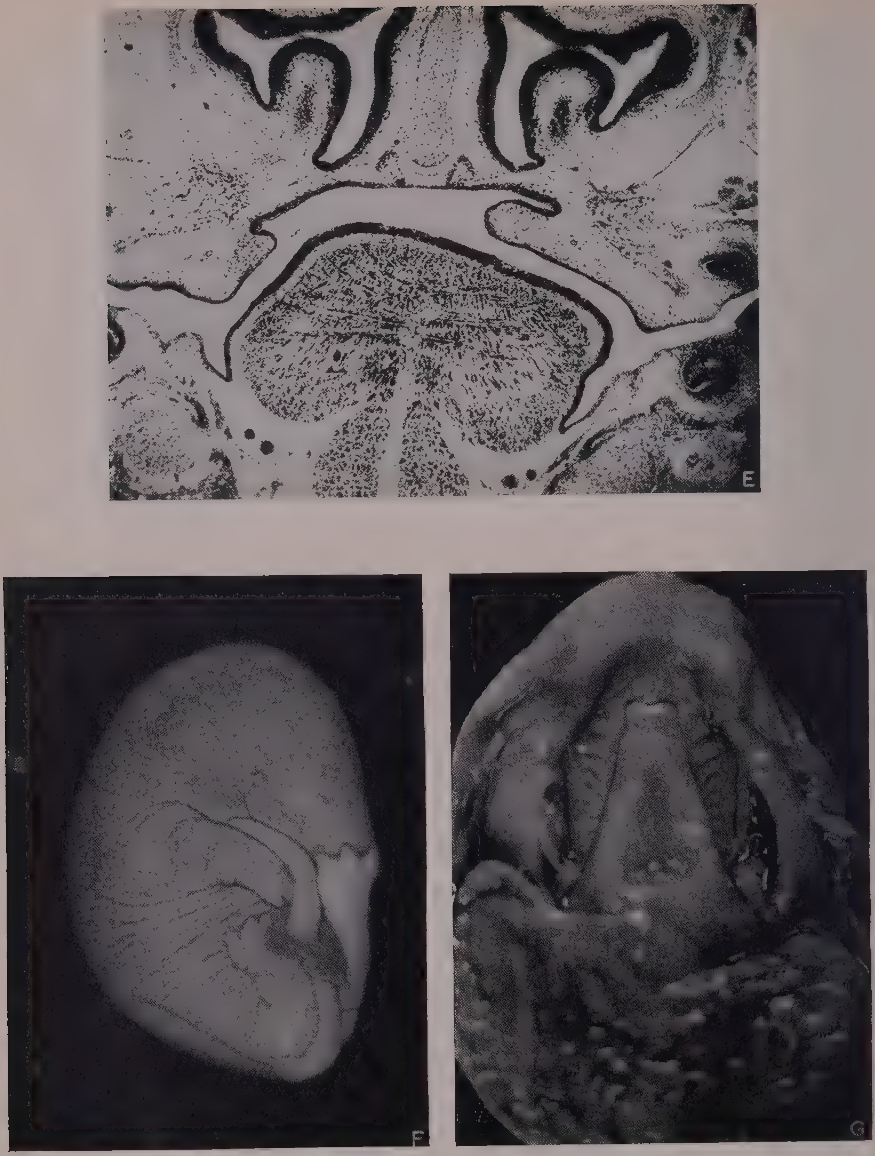


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Plate 2

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EXPLANATION OF PLATES

Plate 1

Fig. A. The palate of an untreated A/Jax embryo, age 15 days 4 hours, showing an intermediate stage of palate closure. ×15.

Fig. B. The palate of a cortisone-treated A/Jax embryo, age 16 days 8 hours. The palatine shelves are still in the vertical position. ×15.

Fig. C. The palate of a cortisone-treated A/Jax embryo, age 15 days 16 hours. The shelves have become horizontal, but do not touch. ×15.

Fig. D. The palate of a cortisone-treated A/Jax embryo, age 16 days 8 hours. The width of the space between the shelves increases as the head grows larger. ×15.

Plate 2

Fig. E. A cross-section through the palate region of a cortisone-treated A/Jax embryo, age 16 days 8 hours. One shelf is horizontal, the other is in transition from vertical to horizontal. ×65.

Fig. F. An embryo, age 17 days 14 hours, showing effects of puncturing the amniotic sac previous to palate closure. ×8.

Fig. G. The palate of an embryo, age 17 days 14 hours, following puncture of the amniotic sac previous to palate closure. The tongue is wedged tightly between the palatine shelves. ×15.

(Manuscript received 23 : vii : 56)

Total Replacement of the Yolk of Chick Embryos

by C. R. GRAU, N. W. KLEIN, *and* T. L. LAU[1]

From the Department of Poultry Husbandry, University of California, Davis, California

ISOLATION of the avian embryo from its normal food material is an essential step in a fundamental study of embryonic nutrition. With present methods, however, it has not been possible to grow embryos in culture for much longer than one day. In 1932 Waddington described methods of growing explanted embryos on serum clots. Several years later Spratt (1947, 1952) devised simplified media for embryos cultured on agar with which he studied various aspects of morphogenesis and carbohydrate needs of the young embryo. New (1955) has recently reported another technique for growing explanted embryos which allows development to proceed to about 60 hours, 40 hours of which are *in vitro*.

A number of workers have injected various nutrients, antimetabolites, drugs, poisons, and hormones, directly into the yolk sac or other areas of the egg (e.g. Landauer, 1954). This type of study has been very informative, but its scope has been limited by the fact that only limited amounts of material can be injected, and their concentrations may not be uniform in the different parts of the relatively large yolk mass.

The technique described below permits the total replacement of the original egg yolk by yolk material from other eggs without disturbing the normal position of the embryo or its normal growth. This technique was developed as the first necessary step in an attempt to replace the yolk with an artificial medium. If the researcher could manipulate all the nutrients available to the embryo, he could then study directly those egg components which are affected little by the diet of the hen—components such as lipoproteins and their constituent amino acids, phospholipids, sterols, and fatty acids. Other studies which might utilize embryos grown on a defined medium include: the interactions of virus and host; neoplasms; mechanisms of hormone action; and enzyme induction. The exchange of yolk material between one embryo and another should also prove useful in physiology and in genetic studies of such problems as maternal effects, heterosis, and mechanisms of lethal gene action.

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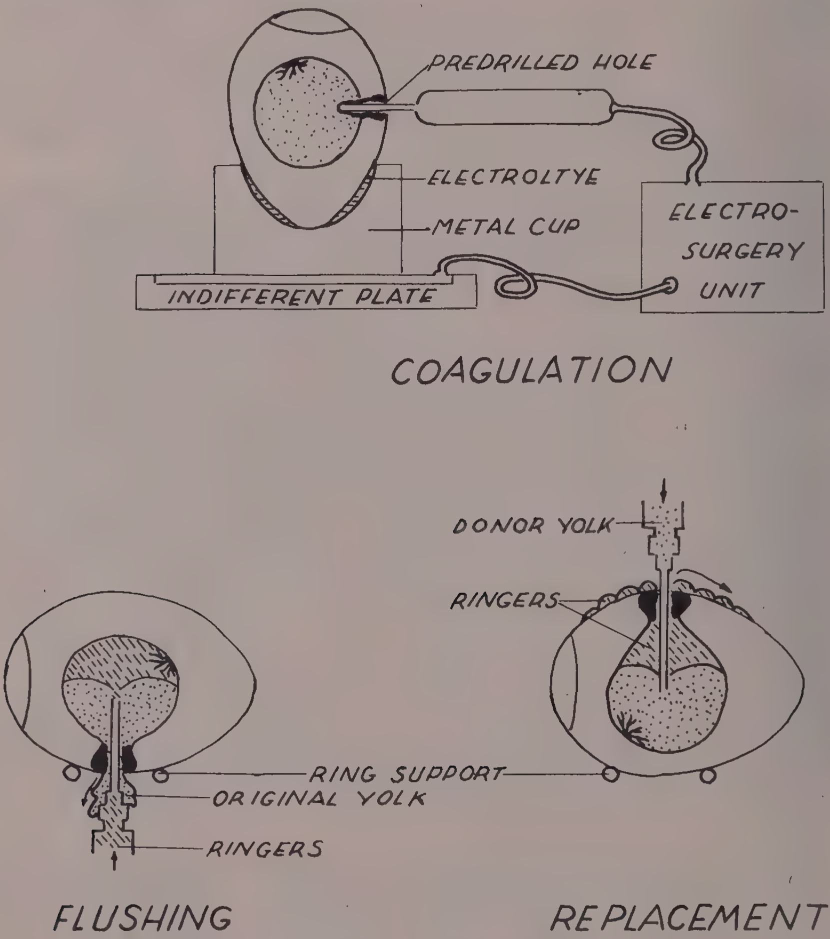
METHODS

The success of the present technique depends primarily upon the formation of a coagulum of albumen which binds the vitelline membrane to the shell membranes. This operation is performed with an electrosurgical unit ('Blendtome', manufactured by the Birtcher Corp.) that combines cutting and coagulation currents. A connexion is made between the yolk and the outside of the shell, allowing either total removal of the yolk by flushing with Ringer's solution, or partial removal by suction. A maximum of approximately 5 ml. of yolk can be removed by direct suction using a syringe; attempts to remove larger volumes result in collapse of the vitelline membrane. When yolk is removed in this way a supplementary hole must be opened into the albumen to allow for change in volume, otherwise air will be drawn into the yolk around the needle.

Fertile eggs from White Leghorn hens on stock diet are incubated for about $2\frac{1}{2}$ days (stages 15–17 of Hamburger & Hamilton, 1951). Sterile technique is used hereafter. The shell is wiped with 70 per cent. ethanol, a hole 3 mm. in diameter is drilled through the shell but not through the shell membrane, and the hole is cleaned with 70 per cent. ethanol. The hole is located on the side of the egg, at the equator or slightly above. The drilled eggs are returned to the incubator where they are kept with the hole downward for a few minutes to orient the embryo away from the hole. Next the egg is supported with the large end upward in a metal cup containing a few drops of an electrolyte, to assure a good electrical contact, and the cup is placed on the indifferent plate of the electrosurgical unit. A straight, sharp, 20-mm. electrode (Birtcher No. 795–B) is inserted quickly through the hole and into the yolk, and the current is turned on for 5–6 seconds (Text-fig. 1, coagulation step). Then, while the current is maintained, the needle is drawn outward in a spiral motion, over a period of about 4 more seconds, until the point is about 5 mm. inside the egg. The current is then turned off and the needle is withdrawn slowly, carrying with it small amounts of coagulated yolk. (The current used is for maximum coagulation (setting 7) and partial cutting (setting 4).) By this method a direct connexion is established between the inside of the yolk and the outside of the shell through a tunnel surrounded by the coagulum.

The egg is next supported in a horizontal position with the hole downward (Text-fig. 1, flushing step) and a hypodermic needle (16 gauge) is used to clear the tunnel of coagulated yolk and albumen fragments. Now a hypodermic needle (20 gauge), clamped firmly and attached to tubing and a reservoir of Ringer's solution containing erythromycin (0·1 mg./ml.) and streptomycin (1·0 mg./ml.), is inserted about 15 mm. into the hole and a pressure of 25–35 cm. of water is used to flush out the yolk. The egg, which is moved occasionally in order to keep the yolk flowing, is kept warm by a heat lamp. Flushing is completed in 5–15 minutes if the preparation is satisfactory. After all the yolk has been flushed out, as determined by clarity of effluent and direct observation before the candling light, the egg is placed hole upward, and the donor yolk, which contains the same antibiotic concentration as the Ringer's solution, is injected through a 16-gauge needle into the yolk cavity (Text-fig. 1, replacement step), thus displacing the Ringer's solution. When the desired amount of yolk (usually 15 ml.) has been injected, the hole is sealed with paraffin and a piece of plastic adhesive tape, and the egg is returned to the incubator.



TEXT-FIG. 1. Three steps in the total replacement of yolk.

RESULTS

Normal growth and development have been observed in embryos after more than 95 per cent. of the original yolk was flushed out with Ringer's solution, and the flushing solution in turn replaced by yolk from other eggs. A number of embryos that were 2½ to 3 days old at the time of replacement have lived until time of hatching, and one actually hatched.

Estimation of the completeness of yolk removal was made by sacrificing embryos just before yolk replacement and determining the carotenoid content of the total egg contents. This was done by extracting exhaustively with acetone, filtering, diluting, and determining the absorbance of the extract at 445 mμ, in comparison to intact eggs. Most of the yolk that was found after flushing was part of the coagulum surrounding the tunnel connecting the yolk cavity with the exterior. This material was denatured and probably would not contribute a significant amount of nutrients to the embryo.

Embryos of various ages up to 5 days have been subjected to the technique, but in none older than 3½ days or younger than 2 days have we yet obtained a successful replacement. In the older embryos the coagulum was not strong enough to withstand the flushing procedure. In unincubated eggs or for the first 2 days of incubation, the embryo is not tightly attached to the vitelline membrane, and is easily washed free during flushing.

Attaching the yolk to the shell membrane is accomplished with little mortality, and can be performed before incubation, or at any time up to 3 days of age. The coagulum is quite strong, and may be used repeatedly as an entrance into the yolk sac. If desired, the yolk can be cut free from the shell after sealing with paraffin by running an L-shaped knife blade around the inside of the hole. The small gas bubbles which are formed in both the yolk and the albumen during coagulation are apparently not harmful to the embryo.

In the course of developing the present technique we used some modifications in which the albumen was removed from a partially opened egg by suction with a syringe. This step could be done with the present method, after the yolk was replaced and the hole sealed, thus permitting replacement of all nutrients available to the embryo.

TABLE 1

Numbers of surviving embryos after total replacement of yolk by yolk from three different sources. The first column of figures represents the number of 3-day-old embryos that survived the operation and were incubated. The remaining columns show the numbers of these embryos that were alive on the days indicated*

Kind of replacement yolk	*Embryo age in days* 3	4	5	11	20
Unincubated infertile	9	3	3	0	0
Unincubated fertile	14	3	3	2	2†
From 5-day embryos	15	8	7	5	1
Totals	38	14	13	7	3

* 38 out of 74 survived the operation. † One of these hatched.

The data presented in Table 1 show how one group of embryos prepared during a 2-day period survived subsequent days. Failures during the operation were caused by unsatisfactory coagulation, which could readily be detected by the presence of albumen in the flushed yolk, or by separation of the embryo from the vitelline membrane, which could be observed by candling. These failures did not entail much loss of effort. In the periods after the operation the greatest losses were those resulting from separation of the extra-embryonic circulation from the vitelline membrane. Most of these failures were detected during the first 24 hours. Embryos that died after the 24-hour period appeared to be normal except for haemorrhagic areas in the embryo itself or in its external circulation.

From the data of Table 1 and others obtained previously, it appears that the chances of survival for more than 24 hours are greater when the donor yolk comes from embryos than when it comes from unincubated or infertile eggs. We believe that survival after this initial period is not much affected by the source of yolk.

SUMMARY

A technique is described in which a coagulum of albumen binds the vitelline membrane to the shell membranes of 3-day chicken embryos, thus allowing the yolk to be flushed out with Ringer's solution. Donor yolk is then injected into the yolk cavity, where it displaces the Ringer's solution. Normal development occurred, including the hatching process, when more than 95 per cent. of the original yolk was replaced by other yolk.

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